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(54) Method of amplifying gene using artificial transposon

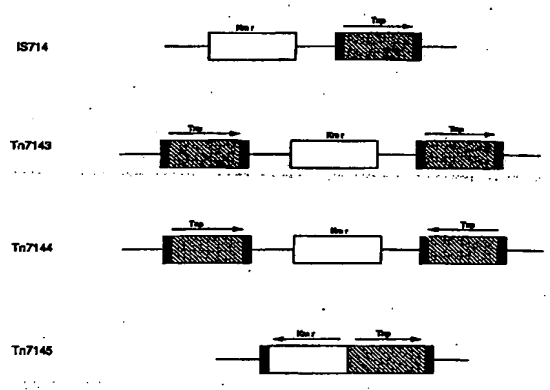
(57) Construction

A method of amplifying a desired gene in a chromosome of a coryneform bacterium, which comprises forming an artificial transposon in which a drug resistance gene and the desired gene are inserted into an insertion sequence of the coryneform bacterium, and introducing said artificial transposon into the coryneform bacterium.

Effects

In accordance with the method of the present invention, a desired gene can be amplified in a chromosome in coryneform bacteria which are used in the industrial production of amino acids or nucleic acids, and the breeding of the coryneform bacteria can be improved.

Fig. 1



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Description

Field of the Invention

The present invention relates to a method of amplifying a desired gene in a chromosome of a coryneform bacterium using an artificial transposon which is transposable in the coryneform bacterium and to a coryneform bacterium obtained by this method. When the desired gene is a gene that participates in biosynthesis of amino acids or nucleic acids, amino acids or nucleic acids can be produced using the thus-obtained coryneform bacterium. A method of amplifying a desired gene in a chromosome is important in improving the breeding of coryneform bacteria which are used in the industrial production of amino acids or nucleic acids.

Prior Art

Studies for improving the breeding of coryneform bacteria and efficiently producing amino acids or nucleic acids have been assiduously conducted so far. A large number of breeding means using the gene engineering have been reported. The gene manipulation for the breeding of coryneform bacteria has been developed in systems using plasmids or phages. There are, for example, the establishment of transformation using protoplasts [J. Bacteriol., by Katsumata R., Ozaki A., Oka T. and Furuya A., 159, 306 - 311, (1984); and J. Bacteriol, by Santamaria R. I., Gil J. A. and Martin J. F., 161, 463 - 467 (1985)], the development of various vectors [Agric. Biol. Chem., by Miwa K., Matsui H., Terabe M., Nakamori S., Sano K. and Momose H, 48, 2901 - 2903 (1984); J. Bacteriol., by Katsumata R., Ozaki A., Oka T. and Furuya A., 159, 306 - 311 (1984); J. Gen. Microbiol., by Santamaria R. I., Gil J. A., Mesas J. M. and Martin J. F., 130, 2237 - 2246 (1984); Gene, by Yeh P., Oreglia J., Prevotos F. and Scicard A. M., 47, 301 - 306 (1986); and Appl. Microbiol. Biotechnol., by Patek M., Nesvera J. and Hochmannova J., 31, 65 - 69 (1989)], the development of a method of controlling gene expression (Bio/Technology, by Tsuchiya M. and Morinaga Y., 6, 428 - 430 (1988)], and the development of cosmids [Gene, by Miwa K., Matsui K., Terabe M., Ito K., Ishida M., Takagi H., Nakamori S. and Sano K., 39, 281 - 286 (1985)]. The cloning of genes derived from coryneform bacteria was reported in Nucleic Acids Res., by Matsui K., Sano K. and Ohtsubo E., 14, 10113 - 10114 (1986); J. Bacteriol, by Follettie M. T. and Shinskey A. J., 167, 695 - 702 (1986), Nucleic Acids Res., by Mateos L. M., Del R. G., Aguilar A. and Martin J. F., 15, 10598 (1987); Nucleic Acids Res., by Mateos L. M., Del R. G., Auilar A. and Martin J. F., 15, 3922 (1987); Nucleic Acids Res., by Melumbres M., Mateos L. M., Guerrero C. and Martin J. F., 16, 9859 (1988); Agric. Biol. Chem., by Matsui K., Miwa K. and Sano K., 52, 525 - 531 (1988); Mol. Microbiol., by Peoples O. P., Liebl W., Bodis M., Maeng P. J., Follettie M. T., Archer J. A. and Shinskey A. J., 2, 63 - 72 (1988); Mol. Gen. Genet., by Eikmanns B. J., Follettie M. T., Griot M. U., Martin U. and Shinskey A. J., 218, 330 - 339 (1989); and Gene, by O'Regan M., Thierbach G., Bachmann B., Vgilleval D., Lepage P., Viret J. F. and Lemoine Y., 77, 237 - 251 (1989). The increase in the yields of various amino acids was reported in Agric. Biol. Chem., by Sano K., Miwa K. and Nakamori S., 51, 597 - 599 (1987).

Recently, transposable elements of coryneform bacteria have been reported [WO 92/02627; WO 93/18151; EP0445385; Japanese Laid-Open Patent Application (hereinafter referred to as "Japanese Kokai") No. 46,867/1994; Mol. Microbiol., by Vertes A. A., Inui M., Kobayashi M., Kurusu Y. and Yukawa H., 11, 739 - 746 (1994); Mol. Microbiol., by Bonamy C., Labarre J., Reyer O. and Leblon G., 14, 571 - 581 (1994); Mol. Gen. Genet., by Vertes A. A., Asai Y., Inui M., Kobayashi M., Kurusu Y. and Yukawa H., 245, 397 - 405 (1994); FEMS Microbiology Letters, by Jagar W., Schafer A., Kalinowski J. and Puehler A., 126, 1 - 6, (1995); and Japanese Kokai No. 107,976/1995).

The transposable element is a DNA fragment that can be transposed in a chromosome, and it is known that this transposable element is present in a broad range of organisms including procaryotes and eucaryotes. Detailed knowledges are given with respect to the eucaryotes such as corns, drosophilae, yeasts and the like, and the procaryotes such as Escherichia coli and the like [Mobile DNA, American Society for Microbiology, Washington D.C. (1989)]. The transposable element of bacteria is grouped into two types, an insertion sequence and a transposon. The insertion sequence is a DNA fragment which has a size of approximately from 760 to 2,000 bp, which has inverted repeats of approximately from 8 to 20 bp at both ends and which encodes transposase, an enzyme necessary for transposition thereinside. Meanwhile, the transposon is a transposable element having the inverted repeats and the transposase as well as a gene such as a drug resistance gene which does not directly participate in transposition performance. The transposon generally includes one in which the drug resistance gene is held between the two insertion sequences and one in which the drug resistance gene is inserted in the insertion sequence. Both of the insertion sequence and the transposon are characterized in that the duplication of the nucleotide sequence of approximately 10 bp is observed in a target gene site having introduced therein the insertion sequence or the transposon [Mobile Genetic Elements, Academic Press, New York, pp. 159 - 221 (1983)].

The transposable element which is currently known includes transposons Tn10 and Tn5 of Escherichia coli and Mu phage which are quite useful in the chromosome gene engineering. For example, it is considered that 1) a transposon is transposed in a chromosomal gene to destroy the gene, repressing the expression of this chromosomal gene, that 2) a promoter sequence is inserted into a transposon to express the chromosomal gene present in the insertion site, and

that 3) a heterologous desired gene or a homologous desired gene is contained in a transposon for transposition to introduce the new gene into a chromosome [Mobile DNA, American Society for Microbiology, Washington D.C., pp. 879 - 925 (1989)].

A transposable element which is an insertion sequence has been recently found in coryneform bacteria, but a transposable element which is a transposon having a drug resistance gene or the like has not been found. It has been possible to produce a transposon in which a kanamycin resistance gene is artificially inserted [WO93/18151; Japanese Kokai No. 107,976/1995; and Mol. Gen. Genet., by Vertes A. A., Asai Y., Inui M., Kobayashi M., Kurusu Y. and Yukawa H, 245, 397 - 405 (1994)] and transpose the same into a chromosome. The artificial transposon produced therein includes one in which a drug resistance gene is held between two insertion sequences (WO93/18151) and one in which a drug resistance gene is inserted in an insertion sequence [Japanese Kokai No. 107,976/1995; and Mol. Gen. Genet., by Vertes A. A., Asai Y., Inui M., Kobayashi M., Kurusu Y. and Yukawa H., 245, 397 - 405 (1994)]. The transposition by multi-copying of such an artificial transposon is not observed or an increase in the number of copies is not satisfactory. Accordingly, a technology for amplification of genes with the use of this transposon which is useful in the industries of amino acids or nucleic acids has not yet been established.

Problems To Be Solved by the Invention

It is an object of the present invention to provide a method, which comprises forming an artificial transposon containing a drug resistance gene and a desired useful gene on the basis of an insertion sequence of a coryneform bacterium, amplifying the desired gene in a chromosome of the coryneform bacterium employed in the industrial production of amino acids or nucleic acids using the above-mentioned artificial transposon.

Another object of the present invention is to provide a coryneform bacterium in which a desired useful gene is amplified in a chromosome.

Another object of the present invention is to provide a method for producing a substance by using a coryneform bacterium in which a desired useful gene is amplified in a chromosome.

Means Taken For Solving the Problems

In order to solve the above-mentioned problems, the present inventors have focused on the fact that a known transposon of Escherichia coli or the like is transposed such that a structure having a drug resistance gene which does not participate in the transposition performance is taken between inverted repeat structures having characteristics of an insertion sequence at both ends. The present inventors have variously constructed artificially a transposon-like sequence having such a structure that a drug resistance gene and a desired gene which do not participate in the transposition performance is inserted between inverted repeats at both ends of an insertion sequence derived from a chromosomal DNA of a coryneform bacterium, and have assiduously conducted studies. As a result, they have found that this transposon-like sequence (artificial transposon) is transposed at good efficiency, and that a gene amplified microorganism in which many copies of the artificial transposon are transposed into its chromosome can be formed at g od efficiency by appropriately selecting a drug resistance gene and determining the drug concentration. These findings have led to the completion of the present invention.

That is, the present invention provides a method of introducing and amplifying a desired gene on a chromosome, which comprises forming an artificial transposon which has a structure that a drug resistance gene and the desired gene are held between an inverted repeat and which is transposable in a coryneform bacterium cell, introducing said artificial transposon into the coryneform bacterium cell, and transposing said transposon into the chromosome of the coryneform bacterium.

In a preferred embodiment of the above method the artificial transposon has a structure that a transposase is further held between the inverted repeat.

As further preferred embodiments, the present invention provides methods of the above kind, wherein the inverted repeat is derived from an insertion sequence of a coryneform bacterium;

a method, wherein the insertion sequence has a base sequence represented by any one of Sequence Nos. 1,5 or 9 of Sequence Table;

a method, wherein the drug resistance gene is a chloramphenicol resistance gene or a tetracycline resistance gene;

a method, wherein the desired gene is a gene that participates in amino acid biosynthesis;

and a method, wherein the desired gene is an aspartokinase gene and/or a dihydropicolinic acid synthetase gene.

Further the invention provides a coryneform bacterium which is formed by transposing the desired gene into the chromosome by any one of the above methods.

Finally, the present invention provides a method of producing an amino acid, which comprises culturing a coryneform bacterium formed by transposing the gene that participates in amino acid biosynthesis into its chromosome by the

method of invention 6 in a culture medium to form and accumulate the amino acid in the culture medium, and recovering said amino acid.

According to a preferred method of producing an amino acid the gene that participates in amino-acid biosynthesis is an aspartokinase gene and/or a dihydropicolinic acid synthetase gene, and the amino acid is lysine.

The inverted repeat referred to in the present invention is preferably ones which exist on the both terminals of a transposable element isolated from a coryneform bacterium. As the examples of the transposable element derived from a coryneform bacterium, insertion sequences listed as the sequence numbers 1, 5 and 9 of the sequence table are known (WO93/18151). The insertion sequence IS714 in the sequence number 1 has a sequence of the sequence number 3 at the 5' terminal and a sequence of the sequence number 4 at the 5' terminal of the reverse strand, which form an inverted repeat. The insertion sequence IS719 in the sequence number 5 has a sequence of the sequence number 7 at the 5' terminal and a sequence of the sequence number 8 at the 5' terminal of the reverse strand, which form an inverted repeat. An inverted repeat can be formed by putting both at the 5' terminal and at the 5' terminal of the reverse strand one kind of sequences selected from a group of the sequence numbers 3, 4, 7 and 8. An inverted repeat can be formed by putting both at the 5' terminal and at the 5' terminal of the reverse strand two kind of sequences selected from a group of the sequence numbers 3, 4, 7 and 8.

The insertion sequence IS903 in the sequence number 9 has a sequence of the sequence number 10 at the 5' terminal and a sequence of the sequence number 11 at the 5' terminal of the reverse strand, which form an inverted repeat. An inverted repeat can be formed by putting both at the 5' terminal and at the 5' terminal of the reverse strand one kind of sequences selected from a group of the sequence numbers 10 and 11. An inverted repeat can be formed by putting both at the 5' terminal and at the 5' terminal of the reverse strand two kind of sequences selected from a group of the sequence numbers 10 and 11.

The inverted repeat of the present invention can be formed with any sequence other than those listed in sequence numbers 3, 4, 7, 8, 10 and 11 which can function in a transposable element.

The drug resistance gene to be inserted into the insertion sequence includes a kanamycin resistance gene, a chloramphenicol resistance gene and tetracycline resistance gene as well as genes which have resistance to various drugs, such as ampicillin resistance gene, methotrexate resistance gene and the like. The drug resistance gene which has a correlation between the degree of drug resistance and the number of copies of the drug resistance gene is preferable.

As the desired gene to be amplified, genes which participate in biosynthesis of various amino acids and nucleic acids can be mentioned. Examples thereof include a glutamic acid dehydrogenase gene for biosynthesis of glutamic acid, a glutamine synthetase gene for biosynthesis of glutamine, an aspartokinase gene (hereinafter aspartokinase is referred to as "AK" . provided that a gene coding for an AK proteln is hereinafter referred to as "lySC", if necessary), a dihydrodipicolinate synthase gene (hereinafter dihydrodipicolinate synthase is referred to as "DDPS", provided that a gene coding for a DDPS proten is hereinafter referred to as "dapA", if necessary), a dihydrodipicolinate reductase gene (hereinafter dihydrodipicolinate reductase is referred to as "DDPR", provided that a gene coding for a DDPR protein is hereinafter referred to as "dapB", if necessary), a diaminopimelate decarboxylase gene (hereinafter diaminopimelate decarboxylase is referred to as "DDC", provided that a gene coding for a DDC protein is hereinafter referred to as "lysA", if necessary). and a diaminopimelate dehydrogenase gene (hereinafter diaminopimelate dehydrogenase is referred to as "DDH", provided that a gene coding for a DDH protein hereinafter referred to as "ddh", if necessary) for biosynthesis of lysine, a homoserine dehydrogenase gene for biosynthesis of threonine, an acetohydroxy acid synthetase gene for biosynthesis of isoleucine or valine, a 2-isopropylmalic acid synthetase gene for biosynthesis of leucine, a glutamic acid kinase gene for biosynthesis of proline or arginine, a phosphoribosyl-ATP pyrophosphorylas g ne for biosynthesis of histidine, a deoxyarabinohepturonic acid phosphate (DAHP) synthetase gene for biosynthesis of aromatic amino acids such as tryptophan, tyrosine and phenylalanine, and a phosphoribosylpyrophosphate (PRPP) amidotransferase gene, an inosine guanosine kinase gene, an inosinic acid (IMP) dehydrogenase gene and a guanylic acid (GMP) synthetase gene for biosynthesis of nucleic acids such as inosinic acid and guanylic acid. Further, genes encoding physiologically active proteins such as interleukin 2, interleukin 6 and the like are also available.

The coryneform bacteria referred to in the present invention include, as described in Bergey's Manual of Determinative Bacteriology, 8th ed., p. 599 (1974), aerobic Gram-positive bacilli, bacteria which are classified to the genus Corynebacterium, bacteria which were once classified to the genus Brevibacterium but now are classified to the genus Corynebacterium [Int. J. Syst. Bacteriol., 41, 255 (1981)], bacterium of the genus Brevibacterium which is quite close to the genus Corynebacterium, and bacteria of the genus Microbacterium. Generally, the following microorganisms which are known as L-glutamic acid-producing bacteria are included in the coryneform bacteria.

Corynebacterium acetoacidophilum

Corynebacterium acetoglutamicum

Corynebacterium callunae

Corynebacterium glutamicum

Corynebacterium lilium (Corynebacterium glutamicum)

Corynebacterium melassecola

Brevibacterium divaricatum (Corynebacterium glutamicum)

Brevibacterium flavum (Corynebacterium glutamicum)

Brevibacterium immariophilum

Brevibacterium lactofermentum (Corynebacterium glutamicum)

Brevibacterium roseum

Brevibacterium saccharolyticum

Brevibacterium thiogenitalis

Brevibacterium ammoniagenes (Corynebacterium ammoniagenes)

Microbacterium ammoniaphilum

Corynebacterium thermoaminogenes

Specifically, the following wild strains and mutant strains derived therefrom are mentioned.

Corynebacterium acetoacidophilum ATCC 13870
Corynebacterium acetoglutamicum ATCC 15806
Corynebacterium callunae ATCC 15991
Corynebacterium glutamicum ATCC 13020
Corynebacterium lilium (Corynebacterium glutamicum) ATCC 15990
Corynebacterium melassecola ATCC 17965
Brevibacterium divaricatum (Corynebacterium glutamicum) ATCC 14020
Brevibacterium flavum (Corynebacterium glutamicum) ATCC 14067
Brevibacterium immariophilum ATCC 14068
Brevibacterium lactofermentum (Corynebacterium glutamicum) ATCC 13869
Brevibacterium roseum ATCC 13825
Brevibacterium saccharolyticum ATCC 14066
Brevibacterium thiogenitalis ATCC 19240
Brevibacterium ammoniagenes (Corynebacterium ammoniagenes) ATCC 6871
Microbacterium ammoniaphilum ATCC 15354
Corynebacterium thermoaminogenes AJ 12340 (FERM BP-1539)

The arrtificial trasposon of the present invention has a structure that a drug resistance gene and a desired gene are held between an inverted repeat and has an ability to transpose in a coryneform bacterium.

The transposase of the present invention is, for example, one which has a nucleotide sequence listed in the sequence numbers 2 or 6 of the sequence table. However the transposase of the present invention also includes ones which has a deltion, an insertion, an addition, a substitution or a inversion of one or more than two amino acid residues on the above sequence as long as it has a transposase activity. The transposase gene of the present invention has, for example, a sequence from the 130th to the 1437th of the nucleotide sequence in the sequence number 1 or a sequence from the 130th to the 1437th of the nucleotide sequence in the sequence number 5. However the transposase gene of the present invention also includes ones which has a deltion, an insertion, an addition, a substitution or a inversion of one or more than two amino acid residues on the above sequence as long as the gene product has a transposase activity.

The transposase gene can be placed inside an artificial transposon. That is, the transposon gene is held between an inverted repeat and placed at the position which does not interfere functions of a drug resistance gene and a desired gene. The transposase gene can be placed outside an artificial transposon. The transposase gene can be carried on a

plasimd having an artificial transposon in one plasmid. The transposon gene can be carried on another plasmid than a plasmid having an artificial transposon in two plasmids. The transposon gene can exist on a chromosome.

The artificial transposon of the present invention can be easily constructed from a transposable element as a starting material.

In the present invention, any insertion sequence can be used so long as it is present in the chromosome of the above-mentioned coryneform bacteria, has a size of approximately from 760 to 2,000 bp, has inverted repeats of approximately from 8 to 20 bp and encodes therein a transposase necessary for transposition. Such an insertion sequence is obtained according to the method disclosed in WO93/18151. That is, a DNA fragment containing an insertion sequence can be obtained by 1) introducing plasmid pEC701 into a coryneform bacterium for transformation, 2) selecting the strain transformed with pEC701 using kanamycin resistance as a marker, 3) spreading the coryneform bacterium containing plasmid pEC701 on an agar plate containing isopropyl-β-thiogalactoside (IPTG) and selecting the thus-grown strain, 4) analyzing the regulatory gene region or the structural gene region of the chloramphenicol acetyl transferase gene in the plasmid contained in the selected strain, and 5) finding the sequence inserted in this gene. Alternatively, the above-mentioned DNA fragment can be obtained by 1) introducing plasmid pEC901 into a coryneform bacterium for transformation, 2) selecting the strain transformed with pEC901 using kanamycin resistance as a marker, 3) incubating the coryneform bacterium containing pEC901 at 30°C and selecting the strain that expresses chloramphenicol resistance even at 30°C, 4) analyzing the cI repressor gene of the plasmid contained in the selected strain, and 5) finding the sequence inserted in this gene.

Specific examples of the insertion sequence of the coryneform bacterium includes three types of insertion sequences represented by Sequence Nos. 1, 5 and 9 of Sequence Table, namely IS714, IS719 and IS903. These nucleotide sequences are not necessarily strict ones. A insertion sequence including an inverted repeat sequence in which a part of bases are replaced with other bases or deleted or a new sequence is inserted or added can be used in the construction of the artificial transposon so long as it serves as an insertion sequence.

A variety of artificial transposons can be constructed on the basis of these insertion sequences. The structures of these artificial transposons are shown in Fig. 1. Of these, the artificial transposon which is used in the present invention has a structure that a drug resistance gene and a desired gene to be amplified are inserted in the insertion sequence.

In case of IS714 shown in the sequence number 1, a restriction enzyme Nhe I site is on the position from 37th to 42nd. The position is suitable for inserting a drug resistance gene and a desired gene to be amplified since the insertion at the position does not interfere the functions of the inverted repeat and transposase.

In case of IS719 shown in the sequence number 5, a restriction enzyme Nhe I site is on the position from 37th to 42nd. The position is suitable for inserting a drug resistance gene and a desired gene to be amplified since the insertion at the position does not interfere the functions of the inverted repeat and transposase.

In case of IS903 shown in the sequence number 9, a restriction enzyme Xcm I site is on the position from 34th to 48th. The position is suitable for inserting a drug resistance gene and a desired gene to be amplified since the insertion at the position does not interfere the functions of the inverted repeat and transposase.

A method of constructing an artificial transposon having inserted therein a gene which is resistant to a drug such as kanamycin (neomycin), chloramphenicol or tetracycline, and a method of constructing an artificial transposon having inserted therein the drug resistance gene and a desired gene useful for production of amino acids or nucleic acids (e.g. aspartokinase) will be described hereinafter, using IS714 as an example. The nucleotide sequence of IS714 is represented by Sequence No. 1 of Sequence Table.

(1) Construction of an artificial transposon containing a kanamycin resistance gene

Plasmid pEC701-IS14 having a sequence of IS714 which is an insertion sequence of Brevibacterium lactofermentum AJ12036 (FERM BP-734) which is a wild strain of a coryneform bacterium (refer to WO93/18151) is cleaved with restriction endonucleases Pvu II and Eco RI to obtain a fragment of 1.6 kb containing IS714. Meanwhile, a fragment containing IS714 is inserted into a restriction endonuclease Sal I site of plasmid pHSC4 having a temperature-sensitive replication origin which is derived from a coryneform bacterium (refer to Japanese Kokai No. 7,491/1993) to construct plasmid pHIS714. The above-obtained fragment of 1.6 kb containing IS714 is further inserted in the Sma I site of pHIS714. Thus, plasmid pHTN7141 and pHTN7142 containing the IS714 fragments in opposite directions are constructed as shown in Fig. 2.

Then, pHTN7141 and pHTN7142 are cleaved with restriction endonuclease Pvu II, making it possible to cut out a fragment containing two sequences of IS714 and the sequence of the temperature-sensitive replication origin of pHSC4. Meanwhile, plasmid vector pHSG298 (made by Takara Shuzo) has also two restriction endonuclease Pvu II sites. A fragment of 2.3 kb containing a neomycin phosphotransferase gene (kanamycin resistance gene) can be obtained by cleaving this plasmid vector with restriction endonuclease Pvu II. pHTN7141 and pHSG298 are cleaved with restriction endonuclease Pvu II, and the resulting fragments are then ligated to transform Brevibacterium lactofermentum AJ12036. Plasmid pHTN7143 is obtained from the strain which has kanamycin resistance as shown in Fig. 3.

Plasmid pHTN7144 is obtained from plasmid pHTN7142 and pHSG298 in the above-mentioned manner as shown

in Fig. 4. pHTN7143 and pHTN7144 have a structure that the neomycin phosphotransferase gene is held between two sequences of IS714. Plasmids pHIS714K1 and pHIS714K2 are constructed from plasmid pHIS714 and pHSG298 as control plasmids in the above-mentioned manner as shown in Fig. 5. In pHIS714K1 and pHIS714K2, the directions of the inserted fragments each containing the neomycin phosphotransferase gene are opposite to each other.

In order to minimize the artificial transposon, an artificial transposon is constructed in which a neomycin phosphotransferase gene is inserted into one IS714. In IS714, a restriction endonuclease Nhe I site is present in a position where the transposase function is not impaired. Plasmid pHIS714 is cleaved with restriction endonuclease Nhe I, and the ends thereof are blunted. On the other hand, a neomycin phosphotransferase gene region is cut out from plasmid pUC4K (made by Pharmacia Biotech) with restriction endonuclease Pst I, and the ends thereof are blunted. Both fragments are ligated to obtain desired plasmid pHTN7145 as shown in Fig. 6.

(2) Construction of an artificial transposon containing a chloramphenicol resistance gene

A fragment of approximately 1.1 kb containing a chloramphenicol acetyltransferase gene can be obtained by cleaving plasmid vector pHSG398 (made by Takara Shuzo) with restriction endonuclease Acc II. Then, this Acc II fragment is inserted into a Sma I site of pUC18 (made by Takara Shuzo), and the thus-obtained plasmid is cloned. The desired clone is selected to obtain plasmid pUC18-CM.

Further, in the above-constructed pHIS714K2, the restriction endonuclease Nhe I site of IS714 located in the position which does not impair the transposase function is blunted. A fragment of approximately 1.1 kb containing a chloramphenicol acetyltransferase gene which is cut out from pUC18-CM with Eco RI and Hind III is ligated with this restriction endonuclease Nhe I blunt site of pHIS714K2 to transform Escherichia coli, and the clone having inserted therein the chloramphenicol acetyltransferase gene fragment is selected. The desired plasmid pHTN7151 can be obtained from the resulting clone as shown in Fig. 7.

(3) Construction of an artificial transposon containing a tetracycline resistance gene

A fragment of approximately 1.4 kb containing a tetracycline resistance gene can be obtained by cleaving plasmid vector pBR322 (made by Takara Shuzo) with restriction endonucleases Eco RI and Ava I. In the above-constructed pHIS714K2, the restriction endonuclease Nhe I site of IS714 located in the position which does not impair the transposase function is blunted. The above-formed DNA fragment is ligated with this restriction endonuclease Nhe I blunt site to transform Escherichia coli, and the clone having inserted therein the tetracycline resistance gene fragment is selected. Desired plasmid pHTN7152 can be obtained from the resulting clone as shown in Fig. 8.

(4) Insertion of an aspartokinase gene which is one of lysine biosynthesis genes into the artificial transposon containing the tetracycline resistance gene.

Since pHTN7152 constructed in Fig. 8 has no good restriction endonuclease site in which to insert an aspartokinase gene, pHTN7156 in which an insertion site is newly introduced is constructed as follows.

A fragment of approximately 1.4 kb containing a tetracycline resistance, gene can be obtained by cleaving plasmid vector pBR322 (made by Takara Shuzo) with restriction endonucleases Eco RI and Ava I. This fragment is ligated with a fragment obtained by cleaving plasmid vector pHY300PLK (made by Takara Shuzo) with restriction endonuclease Sma I to transform Escherichia coli, and the clone having inserted therein the tetracycline resistance gene fragment is selected. Plasmid pHY300-TC is obtained from the resulting clone.

Further, in the above-constructed pHIS714K2, the restriction endonuclease Nhe I site of IS714 located in the position which does not impair the transposase function is blunted. A fragment containing a tetracycline resistance gene which is obtained by cleaving pHY300-TC with restriction endonucleases Eco RI and Xba I is ligated with this restriction endonuclease Nhe I blunt site to transform Escherichia coli, and the clone having inserted therein the tetracycline resistance gene fragment is selected. Desired plasmid pHTN7156 is obtained from the resulting clone as shown in Fig. 9.

Subsequently, an aspartokinase gene which is one of lysine biosynthesis genes is inserted into plasmid pHTN7156 as follows.

Plasmid p399AK9B which contains an aspartokinase gene, which is derived from a lysine-producing mutant of Brevibacterium lactofermentum, a coryneform bacterium, and which is desensitized to concerted inhibition of lysine and threonine (refer to WO94/25605) is cleaved with restriction endonuclease Bam HI, and is self-ligated to construct pHSG399AK from which a replication origin that functions in the coryneform bacterium is removed. This pHSG399AK is cleaved with restriction endonucleases Eco RI and Sph I to obtain an aspartokinase gene fragment of approximately 1.7 kb. This fragment is inserted into the restriction endonuclease Bg1 II blunt site of plasmid pHTN7156 having the artificial transposon containing the tetracycline resistance gene to construct plasmid pHTN7156-C as shown in Fig. 9.

(5)Construction of an artificial transposon containing a tetracycline resistance gene and no transposase in a transposon unit

Plasmid pHIS714 is cleaved with restriction endonucleases Nhe I and Xba I to obtain a fragment containing a gene encoding a transposase. This DNA fragment is introduced into an Xba I site of plasmid vector pUC19 to construct plasmid TnpL/pUC19.

Further, TnpL/pUC19 is cleaved with restriction endonucleases Mro I and Xba I to delete a sequence including a termination codon of IS714 and a 3'-side inverted repeat (IR). A synthetic double-stranded DNA which is designed to reintroduce the terminal codon is inserted into the above-cleaved portion through ligation. In this manner, a transposase gene which is not held between an inverted repeat is obtained.

Subsequently, this ORFL/pUC19 is cleaved with restriction endonucleases Sma I and Xba I to obtain a gene fragment of approximately 1.5 kb containing the transposase. This transposase gene fragment is inserted into a portion of plasmid vector pHY300PLK obtained by removing a sequence between Sma I and Xba I sites thereof, and is then cut with restriction endonucleases Eco RI and Kpn I. The Eco RI and Kpn I fragment is end blunted. Meanwhile, plasmid vector pHSG398 is partially digested with restriction endonuclease Pvu II to delete a fragment containing a multi-clonng site and ligated to the above-obtained transposase gene fragment. Thus plasmid pORF1 can be constructed (Fig. 10).

On the other hand, the Nhe I - Xba I cleavage fragment of plasmid pHIS714 which contains a transposase gene is obtained, end-blunted, and transduced into the end-blunted Pst I site of plasmid vector pUC19 to construct plasmid Tnp (Pst)/pUC19.

The transposase gene of this Tnp(Pst)/pUC19 is subjected to the partial base substitution using a U. S. E. Mutagenesis Kit (made by Pharmacia Biotech). The base substituted is G which is the 288th base in the sequence of IS714. This base G is replaced with C. This base-substituted plasmid is designated as Tnp(Pst)M/pUC19. The structure of Tnp(Pst)M/pUC19 is shown in Fig. 11. * indicates the introduced mutation.

The transposition of a transposable element is controled by a variety of systems. Examples of the control include the followings (Mobile DNA, American Society for Microbiology, Washington D. C. (1989)).

1) An inhibitor gene or a repressor gene of a transposase is located next to a transposase gene insied a transposable element (e.g. Tn 3).

2) Two ORF exist in one frame. The one closer to the 3' termeinal encodes. Tranlational frameshift between the two ORF takes place at a low frequency to make the two ORF translated throughout, which express a transposase (e.g. IS1)

3) In an ORF encoding a transposase exists another translational initiation codon (ATG, GTG) and translation starts from the codon to express an inhibitior (e.g. Tn5 (IS50)).

Meanwhile, in IS714 exists one ORF which corresponds to almost the entire length of IS714 and no other ORF is found. This indicates the possibility that IS714 has an ORF encoding a transposase like Tn5 and that an inhibitior is translated from another initiation codon in the ORF. Result of searching a promoter like sequence reveals a possibility that the sequence GTG from the 286 th to the 288th is the initiation codon of an inhibitor. The mutation introduced on plasmid Tnp(Pst)M/pUC19 is designed not to start the translation of the inhibitor.

The sequence between restriction endonuclease Sma I and Nae I sites present in the transposase first half gene is deleted from pORF1. The transposase first half gene fragment obtained by cleaving Tnp(Pst)M/pUC19 with restriction endonucleases Sma I and Nae I is inserted into the above-deleted portion through ligation to construct pORF2.

The sequence between the Sma I and Xba I sites is deleted from pORF2, and the resulting fragment is end-blunted. A DNA fragment containing a tryptophan operon attenuator is obtained by cleaving pBSF2-SD7 with restriction endonucleases Nae I and Hind III, and is then end-blunted. The former fragment is ligted with the latter fragment. The thus-constructed plasmid is designated pORF3.

pORF3 is cleaved with restriction endonucleases Sal I and Bpu II02I to delete the transposase first half gene fragment. The transposase first half gene fragment obtained by cleaving Tnp(Pst)/pUC19 with restriction endonucleases Sal I and Blu II02I is inserted into the above-deleted portion by ligation to construct pORF4 as shown in Fig. 11.

TnpL/pUC19 is cleaved with Sac I, and is then digested with BAL 31 nuclease at 30°C for 20 minutes to delete a sequence near the initiation codon of the transposase gene from the upstream side. After that, the transposase gene fragment is cut out using the Sph I site, and is ligated with pHSG398 which is cleaved with Sma I and Sph I. The thus-constructed plasmid is designated delTnp5/398.

This delTnp5/398 is cleaved with restriction endonucleases Knp I and Hind III, and the resulting transposase first half gene fragment is end-blunted. Then, plasmid vect r pKK233-2 (made by Pharmacia Biotech) is cleaved with Nco I and Hind III, and is end-blunted. The former fragment is ligated with the latter fragment to construct pTrc-ORF.

pTrc-ORF is cleaved with Ssp I and Bpu 1102I to form a fragment containing Trc promoter and the transposase first

half gene. pORF3 is cleaved with Xba I, end-blunted, and further cleaved with Bpu II02I to delete the transposase first half gene fragment. The above-formed fragment is ligated with this deleted pORF3 to construct pORF7 as shown in Fig. 12.

The transposase first half gene fragment obtained by cleaving delTnp5/39 with restriction endonucleases Kpn I and Hind III is cloned between the KpnI and Hind III sites of plasmid vector pUC18. The portion between the Bsm I and Nae I sites of this plasmid delTnp5/18 is deleted, and the fragment is ligated with the transposase first half gene fragment obtained by cleaving Tnp(Pst)M/pUC19 with restriction endonucleases Bsm I and Nae I to construct delTnp5M/18.

This delTnp5M/18 is cleaved with Kpn I and Hind III, and the resulting transposase first half gene fragment is end-blunted. pKK233-2 is cleaved with Nco I nd Hind III, and the resulting fragment is end-blunted. These fragments are ligated with each other to construct pTrc-TnpM.

pORF8 is constructed from pTrc-TnpM and ORF3 by the same method of constructing pORF7 from pTrc-Tnp (Fig. 13).

Plasmids for being introduced into a coryneform bcterium are constructed using the above-mentioned plasmids pORF3, pORF4, pORF7 and pORF8. The construction of pORF41 from pORF3 is described below.

First, pHIS714 is cleaved with Nhe I and Sac II to delete the major part of the transposase gene. A double-stranded synthetic DNA designed to introduce a cloning site is inserted into the above-deleted portion to construct pHTN7160.

pHTN7160 is cleaved with restriction endonuclerase Kpn I, end-blunted, and then cleaved again with Bgl I to obtain a fragment containing inverted repeats (IR) on both sides of IS714 and a temperature-sensitive replication origin that functions within a coryneform bacterium.

pORF3 is cleaved with restriction endonuclease Ear I, end-blunted, and then cleaved again with Bgl I. The above-mentioned fragment of pHTN7160 is inserted therein to construct pORF41-pre.

Then, pORF41-pre is cleaved with Eco RV which is located between IRs at the both terminals of IS714. An Eco RI-Ava I fragment which contains a Tc resistance gene of pBR322 is end-blunted and ligated with the Eco RV-cleaved fragment to construct pORF41 as shown in Fig. 14.

The above-mentioned method is repeated to construct pORF31 from pORF4 through pORF31-pre, pORF71 from pORF7 through pORF71-pre, and pORF81 from pORF8 through pORF81-pre, respectively.

pORF3 is cleaved with Xba I and Ear I, end-blunted, and self-ligated to construct pORFC0 containing no transposase gene (Fig. 15).

pORFC2 is constructed from pORFC0 through pORFC2-pre in the same manner as in constructing pORF41 form pORF3.

These finally constructed plasmids have the structural gene of the transposase, the Cm resistance gene, the replication origin that functions within E. coli, the temperature-sensitive replication origin that functions within a coryneform bacterium and the Tc resistance gene held between IRs of IS714, provided pORFC2 has no structural gene of the transposase.

The unit containing IRs on both ends of IS714 and the Tc resistance gene is designated as transposon unit Tn7162. IS714 itself or the above described Tn7152 and the like have a structural gene of a transposase within a region which is able to transpose, while Tn7162 is characterized in the structure that it does not have a structural gene of a transposase within a region which is able to transpose. It is considered that Tn7162 is transposed by a transposon expressed from a transposon gene which is located outside the unit and is on the vector carrying Tn7162 (Fig. 16). Or it is considered that Tn7162 transposes by a transposase expressed from a transposase gene on a chromosome.

Next the construction of a plasmid for coryneform bacteria which contains a transposase gene and no transposon unit is explained.

Plasmid pHIS714K1 is cleaved with EcoO 109I and Mro I to delete IS714, and is then self-ligated to construct pHIS714Kdel.

Meanwhile, pORF3 is cleaved with restriction endonuclease Ear I, end-blunted, and cleaved again with Bgl I. pHIS714Kdel is cleaved with restriction endonuclease Kpn I, end-blunted, and then cleaved again with Bgl I to form a fragment which contains a temperature-sensitive replication origin functioning within coryneform bacteria. The thus-formed fragments are ligated with each other to construct pORF40 as shown in Fig. 17.

This method is repeated to construct pORF30 from pORF4, pORF70 from pORF 7, pORF80 from pORF 8 and pORFC1 from pORFC0 respectively.

With respect to insertion sequences of the coryneform bacteria such as IS719 and IS903 having the nucleotide sequences represented by Sequence Nos. 5 and 9 of Sequence Table which are defferent from the above-mentioned IS714, artificial transposons can be constructed by inserting drug resistance genes such as a chloramphenicol resistance gene and a tetracycline resistance gene, and desired genes such as an aspartokinase gene in appropriate restriction endonuclease sites outside the regulatory gene region and the structural gene region of the transposas gene in the insertion sequence. When there is no appr priate restriction endonuclease site outside th regulatory gene region and the structural gene region of the transposase gene, an appropriate restriction endonuclease site may be prepared in advance in a region which does not inhibit the transposase function by modifying the insertion sequence by partial specific mutation of bases using polymerase chain reaction (PCR), or by gene insertion with a synthetic DNA oligonu-

cleotide (adapter).

The thus-constructed artificial transposon is introduced into a host coryneform bacterium through an appropriate vector, for example, a plasmid. A plasmid in which to contain an artificial transposon is not particularly limited. A plasmid derived from coryneform bacteria is usually employed. Examples of the plasmid include pHM1519 [Agric. Biol. Chem., 48, 2901 - 2903 (1984)], pAM330 [Agric. Biol. Chem., 48, 2901 - 2903 (1984)], and drug resistance gene-containing plasmids obtained by improving the above-mentioned plasmids. In order to amplify the artificial transposon introduced in a chromosome at good efficiency, it is advisable to use the plasmid having the temperature-sensitive replication origin as mentioned in (1) [refer to Japanese Kokai No. 7,491/1993].

The plasmid containing the artificial transposon may be introduced into the coryneform bacterium by the protoplast method [Gene, 39, 281 - 286 (1985)] or the electroporation method [Bio/Technology, 7, 1067 - 1070 (1989)].

The artificial transposon may be introduced into a chromosome of a coryneform bacterium through the temperature-sensitive plasmid by transforming the coryneform bacterium with the plasmid constructed, incubating the transformant at 25°C at which the plasmid can be replicated to amplify the artificial transposon-containing plasmid to from sc res to hundreds of copies per cell and introduced into the chromosome, and then conducting incubation at 34°C to remove extra plasmids. The gene amplification is conducted in the chromosome at good efficiency by this method. A normal plasmid can be used instead of the temperature-sensitive plasmid. However, it is difficult, in many cases, to remove extra plasmids after the introduction of the artficial transposon into the chromosome. Further, there is also a method in which an artificial transposon is introduced into a coryneform bacterium using a DNA fragment of an artificial transposon alone or a plasmid vector which cannot be replicated in coryneform bacteria (for example, a plasmid vector which is replicated in Escherichia coli)[Japanese Kokai No. 107,976/1995; and Mol. Gen. Genet., by Vertes A. A., Asai Y., Inui M., Kobayashi M., Kurusu Y. and Yukawa H., 245, 397 - 405 (1994)]. However, in this method, the DNA fragment cannot be amplified within the host strain after the transformation, and the efficiency of transposition into the host chromosome is quite bad.

The strain in which the desired gene is introduced into the chromosome or the strain in which the desired gene is amplified in the chromosome is selected using the degree of drug resistance of the drug resistance gene which is introduced together with the desired gene. The drug resistance gene to be used includes a kanamycin resistance gene, a chloramphenicol resistance gene, a tetracycline resistance gene, an ampicillin resistance gene, a methotrexate resistance gene and the like. The drug resistance gene in which the degree of resistance is correlated with the number of copies of the drug resistance gene is most preferable. That is, it is possible to obtain the strain in which the desired gene is amplified in the chromosome from the clone which can be grown in the presence of the drug having a higher concentration.

After the coryneform bacterium is transformed using the plasmid (for example, pHTN7156-C) containing the drug resistance gene such as the tetracycline resistance gene or the like and the desired gene such as the desensitized aspartokinase gene or the like and the artificial transposon is transposed into the host chromosome, the number of the transposition copies in the chromosome formed after the transposition can be evaluated by the following method.

The transformant is incubated overnight at 25°C in a CM2G liquid medium containing a selected drug such as tetracycline (Tc) or the like at an appropriate concentration (from 1 to 20 μg/ml in the case of Tc), 10 g/liter of yeast extract, 10 g/liter of tryptone, 5 g/liter of glucose and 5 g/liter of NaCl. The culture is appropriately diluted with a 0.9% NaCl solution, and is spread on the CM2G agar medium containing the appropriate concentration of the drug in an amount of 100 μl. The resulting culture is incubated at 34°C. Several clones are selected randomly from many colonies formed. The chromosomal DNA is prepared, completely digested with various appropriate restriction endonucleases including Pvu II, and subjected to agarose gel electrophoresis. The fragments are blotted on a filter of nitrocellulose, nylon or polyvinylidene difluolide (PVDF). This filter is subjected to the southern hybridization using a ^{32}P-labelled tetracycline resistance gene fragment as a probe to detect the number of bands which are hybridized with this probe.

The transformant in which the desired gene is amplified in the thus-obtained chromosome may be incubated using a method and conditions which are ordinarily employed. The culture medium for the incubation is an ordinary culture medium containing a carbon source, a nitrogen source, an inorganic ion and the like. It is advisable that organic micronutrients such as vitamins, amino acids and the like be added as required. Examples of the carbon source include carbohydrates such as glucose and sucrose, organic acids such as acetic acid, and alcohols such as ethanol. Examples of the nitrogen sources include ammonia gas, aqueous ammonia, and ammonium salts. Examples of the inorganic ion include a magnesium ion, a phosphoric acid ion, a potassium ion, and an iron ion. These sources are used as required.

The incubation is conducted aerobically for from 1 to 7 days while controlling the pH to the range of from 5.0 to 8.5 and the temperature to the range of from 15°C to 37°C. The gene is amplified using the artificial transposon, with the result that the efficiency of producing the desired useful substance is increased and the desired substance is produced and accumulated inside or outside the cultured strain. The desired substance can be collected from the culture by a known method.

Examples

The present invention will be illustrated more specifically by referring to the following Examples.

Example 1

Construction of an artificial transposon containing a kanamycin resistance gene using IS714

Plasmid pEC701-IS14 having a sequence of IS714 which is an insertion sequence of a coryneform bacterium was cleaved with restriction endonucleases Pvu II and Eco RI to obtain a fragment of 1.6 kb containing IS714. Brevibacterium lactofermentum AJ12684 containing plasmid pEC701-IS14 was listed as deposited at the National Institute of Bioscience and Human Technology of the Agency of Industrial Science and Technology, Ministry of Industrial Trade and Industry (1-3, Higashi 1 Chome Tsukuba-shi Ibaraki-ken 305, Japan) under deposit No. FERM P-12863 on March 10, 1992, and was transferred to the deposition based on Budapest Treaty on March 9, 1993. Deposit No. BP-4232 is allotted thereto.

Meanwhile, temperature-sensitive plasmid pHSC4 was digested with a restriction endnuclease SalI and made blunt by the treatment with the Klenow fragment. The restriction endonuclease Sal I site of temperature-sensitive plasmid pHSC4 is located in the region which does not participate in the replication. Also a fragment containing IS714 was end-blunted by the treatment with the Klenow fragment. The resulting fragment was then inserted into the restriction endonuclease Sal I site through the ligation to produce plasmid pHIS714 as shown in Fig. 2. Escherichia coli AJ12571 containing plasmid pHSC4 was listed as deposited at the National Institute of Bioscience and Human Technology of th Agency of Industrial Science and Technology, Ministry of Industrial Trade and Industry (1-3, Higashi 1 Chome Tsukubashi Ibaraki-ken 305, Japan) under FERM P-11763 on October 11, 1990, and was transferred to the deposition based on the Budapest Treaty on August 26, 1991. Deposit No. BP-3524 is allotted thereto.

The above-obtained fragment of 1.6 kb containing IS714 was end-blunted by the treatment with the Klenow fragment, and was inserted into the Sma I site of this pHIS714 through the ligation to construct plasmids pHTN7141 and pHTN7142 as shown in Fig. 2. The analysis by the restriction endonuclease cleavage revealed that the two IS714 fragments were inserted in the same direction in plasmid pHTN7141 but in the opposite directions in plasmid pHTN7142.

Fragments each containing two IS714 sequences and the sequence of the temperature-sensitive replication origin in the coryneform bacterium of pHSC4 can be cut out by cleaving pHTN7141 and pHTN7142 with restriction endonuclease Pvu II. On the other hand, plasmid vector pHSG298 (made by Takara Shuzo) has also two restriction endonuclease Pvu II sites. Thus, a fragment of 2.3 kb containing a neomycin phosphotransferase gene (kanamycin resistance gene) can be obtained by cleaving pHSG298 with restriction endonuclease Pvu II.

pHTN7141 and pHSG298 were cleaved with restriction endonuclease Pvu II, and were then ligated with each other to transform Brevibacterium lactofermentum AJ12036. Plasmid pHTN7143 was obtained from the transformant strain which was resistant to 25 μg/ml of kanamycin (Km) as shown in Fig. 3. Brevibacterium lactofermentum AJ12826 containing plasmid pHTN7143 was listed as deposited at the National Institute of Bioscience and Human Technology of the Agency of Industrial Science and Technology, Ministry of Industrial Trade and Industry (1-3, Higashi 1 Chome Tsukubashi Ibaraki-ken 305, Japan) based on the Budapest Treaty on March 9, 1993. Deposit No. Bp-4231 is allotted thereto.

Plasmid pHTN7144 was obtained from pHTN7142 and pHSG298 in the above-mentioned manner as shown in Fig. 4. pHTN7143 and pHTN7144 had a structure that a neomycin phosphotransferase gene was held between two IS714 sequences. Further, plasmids pHIS714K1 and pHIS714K2 were prepared from plasmid pHIS714 and pHSG298 as control plasmids as shown in Fig. 5. In pHIS714K1 and pHIS714K2, the insertion fragments each containing the neomycin phosphotransferase gene were located in opposite sites.

In order to minimize the artificial transposon, an artificial transposon was constructed in which a neomycin phosphotransferase gene was inserted into one IS714 sequence.

The restriction endonuclease Nhe I site is present in a position of IS714 which does not impair the transposase function. Therefore, plasmid pHIS714 was cleaved with restriction endonuclease Nhe I, and the ends thereof were blunted. Meanwhile, the neomycin phosphotransferase gene region was cut out from plasmid pUC4K (made by Pharmacia Biotech) with restriction endonuclease Pst I, and the ends thereof were blunted. These fragments were ligated with each other, and the resulting plasmid was designated pHTN7145 as shown in Fig. 6.

Escherichia coli AJ13128 containing plasmid pHTN7145 was listed as deposited at the National Institute of Bioscience and Human Technology of the Agency of Industrial Science and Technology, Ministry of Industrial Trade and Industry (1-3, Higashi 1 Chome Tsukuba-shi Ibaraki-ken 305, Japan) under FERM P-15011 on June 29, 1995, and was transferred to the deposition based on the Budapest Tr aty on May 16, 1996. Deposit No. BP-5537 is allotted thereto.

Evaluation of transposition performance of artificial transposons

The transposition performance of the thus-obtained artificial transposons was evaluated as follows.

Brevibacterium lactofermentum AJ12036 was transformed with plasmid pAJ43 having the chloramphenicol acetyltransferase gene to produce Brevibacterium lactofermentum AJ 11882. Brevibacterium lactofermentum AJ11882 was transformed with plasmid pHTN7145 containing the artificial transposon so that plasmid pAJ43 and plasmid pHTN7145 were coexistent in this Brevibacterium lactofermentum. Escherichia coli AJ11882 containing pAJ43 was listed as deposited at the National Institute of Bioscience and Human Technology of the Agency of Industrial Science and Technology, Ministry of Industrial Trade and Industry (1-3, Higashi 1 Chome Tsukuba-shi Ibaraki-ken 305, Japan) under FERM P-6517 on April 28, 1982, and was transferred to the deposition based on the Budapest Treaty on May 22, 1982. Deposit No. BP-136 is allotted thereto.

The above-obtained Brevibacterium lactofermentum containing pHTN7145 and pAJ43 coexistently was incubated overnight at 25°C in a CM2G culture medium containing 25 μg/ml of kanamycin (Km), 5 μg/ml of chloramphenicol (Cm), 10 g/liter of yeast extract, 10 g/liter of tryptone, 5 g/liter of glucose and 5 g/liter of NaCl while being shaken. The culture solution was then appropriately diluted, spread on a CM2G agar medium containing 25 μg/ml of Km and 5 μg/ml of Cm, and incubated at 34°C. Plasmids were extracted from 100 strains among the colonies formed, and the sizes thereof were examined through electrophoresis. Of these, three are different with respect to the molecular weights from both plasmids pHTN7145 and pAJ43. They were plasmids of which the molecular weight was the total of the molecular weights of pAJ43 and the artificial transposon.

When these plasmids were analyzed through the restriction endonuclease cleavage, it was found that the sequence in pHTN7145 was inserted into pAJ43. With respect to one of these plasmids, the nucleotide sequence in the vicinity of the portion inserted in pAJ43 and the insertion fragment was determined by the dideoxy method. Consequently, it was identified that the sequences of both ends of the artificial transposon were present, and the target sequence GGTTTATT (Sequence No. 12) on pAJ43 which underwent the insertion was duplicated.

From these results, it was found that when the transposon structure was taken in which the gene that does not participate in the transposition performance (neomycin phosphotransferase gene) was inserted in one IS714 sequence, it was transposed like a transposon with this structure stored.

Evaluation of a transposition frequency of an artificial transposon

Brevibacterium lactofermentum AJ12036 was transformed with pHTN714K1, a control plasmid, pHTN7143, pHTN7144 and pHTN7145 and the frequency of transposition of the artificial transposon into the host chromosome was evaluated. pHTN7143, pHTN7144 and pHTN7145 all contained the artificial transposon.

Each of the transformants was incubated overnight at 25°C in the above-mentioned CM2G liquid medium containing 25 μg/ml of Km. Then, the culture was approximately diluted with a 0.9% NaCl solution, and was spread on CM2G agar medium containing 25 μg/ml of Km in an amount of 100 μl. The resulting substance was incubated at 34°C and 25°C, and the frequency at which the Km resistance strain was appeared at each temperature was measured from the number of colonies. The number of colonies at 34°C was divided by the number of colonies at 25°C. The resulting value was defined as the transposition frequency.

The results are shown in Table 1.

Table 1

Transposable element or artificial transposon	Transposition frequency	Relative ratio
IS714	1.85×10^{-3}	1
Tn7143	3.52×10^{-3}	1.9
Tn7144	2.38×10^{-3}	1.3
Tn7145	2.08×10^{-2}	11.2

From the above-mentioned results, it is found that artificial transposons Tn7143 (contained in pHTN7143) and Tn7144 (contained in pHTN7144) had the frequency of transposition into the host chromosome which was only 1 or 2 times as high as that of IS714 (contained in pHIS714K1) as a control plasmid, but that artificial transposon Tn7145 (contained in pHTN7145) was approximately 11 times and therefore it was a quite efficient artificial transposon.

Example 2

Construction of an artificial transposon containing a chloramphenicol resistance gene using IS714

Plasmid vector pHSG398 (made by Takara Shuzo) was cleaved with restriction endonuclease Acc II to obtain a fragment of approximately 1.1 kb containing a chloramphenicol acetyltransferase gene. This Acc II fragment was inserted into a Sma I site of pUC18 (made by Takara Shuzo), and cloned. That is, a desired clone was selected from Escherichia coli transformant which had been grown in an L-medium containing 25 μg/ml of Cm, 100 μg/ml of ampicillin (Ap), 10 g/liter of tryptone, 5 g/liter of yeast extract and 5 g/liter of NaCl. The plasmid was designated pUC18-CM.

Further, a fragment of approximately 1.1 kb which was cut out from pUC18-CM with Eco RI and Hind III and which contained a chloramphenicol acetyltransferase gene was end-blunted. In pHIS714K2 constructed in Example 1, the restriction endonuclease Nhe I site of IS714 located in the position which does not impair the transposase function was end-blunted by the treatment with the Klenow fragment. The above-mentioned fragment was ligated with this restriction endonuclease Nhe I site to transform Escherichia coli. Colonies which were grown on an L-agar-medium containing 25 μg/ml of Cm and 50 μg/ml of Km were picked up. A clone having inserted therein the chloramphenicol acetyltransferase gene fragment was selected. The plasmid contained in this clone was designated pHTN7151 as shown in Fig. 7.

Escherichia coli AJ13129 obtained by transforming Escherichia coli HB101 with plasmid pHTN7151 was listed as deposited at the National Institute of Bioscience and Human Technology of the Agency of Industrial Science and Technology, Ministry of Industrial Trade and Industry (1-3, Higashi 1 Chome Tsukuba-shi Ibaraki-ken 305, Japan) under FERM P-15012 on June 29, 1995, and was transferred to the deposition based on the Budapest Treaty on May 16, 1996. Deposit No. BP-5538 is allotted thereto.

Evaluation of the number of copies in the chromosome formed by the transposition of the artificial transposon

Brevibacterium lactofermentum AJ12036 was transformed with pHTN7151, and the number of copies of the artificial transposon in the chromosome which were formed by the transposition of the artificial transposon into the host chromosome was evaluated by the following method.

The resulting transformant was incubated overnight at 25°C in the above-mentioned CM2G liquid medium containing 3 μg/ml of Cm, appropriately diluted with a 0.9% NaCl solution, and spread on a CM2G agar medium containing 3 μg/ml of Cm in an amount of 100 μl. The resulting substance was incubated at 34°C. A kanamycin-sensitive clone was selected from colonies appeared. This clone was incubated overnight at 30°C in the above-mentioned CM2G liquid medium containing 3 μg/ml of Cm, appropriately diluted with a 0.9% NaCl solution, and spread on the above-mentioned CM2G agar medium containing 6 μg/ml of Cm in an amount of 100 μl. The resulting substance was incubated at 30°C, and some clones were randomly selected from colonies formed. A chromosomal DNA was prepared from each of the clones, completely digested with restriction endonuclease Pvu II, subjected to agarose gel electrophoresis, and blotted on a polyvinylidene difluoride (PVDF) filter. This filter was subjected to the southern hybridization using a ^{32}P-labelled chloramphenicol acetyltransferase gene fragment as a probe, and the number of bands hybridized with the probe was measured.

As a result, it was identified that in the three of the four clones randomly selected, two copies of the artificial transposon having the chloramphenicol resistance gene were transposed into the host chromosome.

Example 3

Construction of an artificial transposon containing a tetracycline resistance gene using IS714

Plasmid vector pBR322 (made by Takara Shuzo) was cleaved with restriction endonucleases Eco RI and Ava I to obtain a fragment of approximately 1.4 kb having a tetracycline resistance gene. Then, this Eco RI-Ava I-cleaved fragment was end-blunted by the treatment with the T4 DNA polymerase. In pHIS714K2 constructed in Example 1, the restriction endonuclease Nhe I site of IS714 located in the position which does not impair the transposase function was end-blunted by the treatment with the Klenow fragment. The above-mentioned fragment was ligated with this restriction endonuclease Nhe I site to transform Escherichia coli. Colonies grown in an L-agar-medium containing 25 μg/ml of Tc were obtained, and the clone having inserted therein the tetracycline resistance gene was selected. The plasmid contained in this clone was designated pHTN7152 as shown in Fig. 8.

Escherichia coli AJ13130 obtained by transforming Escherichia coli with plasmid pHTN7152 was listed as deposited at the National Institute of Bioscience and Human Technology of the Agency of Industrial Science and Technology, Ministry of Industrial Trade and Industry (1-3, Higashi 1 Chome Tsukuba-shi Ibaraki-ken 305, Japan) under FERM P-15013 on June 29, 1995, and was transferred to the deposition based on the Budapest Treaty on May 16, 1996. Deposit No. BP-5539 is allotted thereto.

Evaluation of the number of copies in the chromosome formed by the transposition of the artificial transposon

Brevibacterium lactofermentum AJ12036 was transformed using pHTN7152, and the number of copies in the chromosome which were formed by the transposition of the artificial transposon was evaluated as follows.

The transformant was incubated overnight at 25°C in the above-mentioned CM2G liquid medium containing 1.5 μg/ml of Tc, then appropriately diluted with a 0.9% NaCl solution, and spread on the above-mentioned CM2G agar medium containing Tc in the range of from 1.5 μg/ml to 5 μg/ml. The resulting substance was incubated at 34°C. Some clones were randomly selected from the colonies formed. A chromosomal DNA was prepared from each of the colonies, completely digested with restriction endonuclease Pvu II, subjected to agarose gel electrophoresis, and blotted on a polyvinylidene difluoride (PVDF) filter. This filter was subjected to the southern hybridization using a ^{32}P-labeled tetracycline resistance gene fragment as a probe, and the number of bands hybridized with the probe was measured.

Consequently, as shown in Table 2, two or three copies of the artificial transposon having the tetracycline resistance gene were detected at high frequency. Thus, it was identified that the desired multi-copying-type transformant could be obtained at high frequency using the tetracycline resistance gene as the selective drug resistance gene.

Table 2

Tc concentration (μg/ml)	Number of test clones	Number of test clones		
		1 copy	2 copies	3 copies
1.5	6	4	2	0
2.0	4	4	0	0
3.0	4	3	1	0
4.0	6	2	3	1
5.0	6	5	1	0

Brevibacterium lactofermentum AJ13188 which is resistant to 4 μg/ml of Tc and is found to have 3 copies of the artificial transposon on the chromosome was listed as deposited at the National Institute of Bioscience and Human Technology of the Agency of Industrial Science and Technology, Ministry of Industrial Trade and Industry (1-3, Higashi 1 Chome Tsukuba-shi Ibaraki-ken 305, Japan) based on the Budapest Treaty on May 14, 1996. Deposit No. BP-5536 is allotted thereto.

Example 4

Construction of an artificial transposon containing a tetracycline resistance gene and an aspartokinase gene using IS714

An aspartokinase gene which is one of lysine biosynthesis genes was inserted into an artificial transposon containing a tetracycline resistance gene in the following manner.

Plasmid vector pBR322 (made by Takara Shuzo) was cleaved with restriction endonucleases Eco RI and Ava I to obtain a DNA fragment of approximately 1.4 kb containing the tetracycline resistance gene. This Eco RI-Ava I-cleaved fragment was end-blunted by the treatment with the T4 DNA polymerase. The thus-obtained DNA fragment was ligated with a fragment obtained by cleaving plasmid vector pHY300PLK (made by Takara Shuzo) with restriction endonuclease Sma I to transform Escherichia coli. Colonies grown in an L-agar-medium containing 25 μg/ml of Tc were obtained, and the clone having inserted therein the tetracycline resistance gene fragment was selected. The plasmid of this clone was designated pHY300-TC.

Further, a fragment obtained by cleaving pHY300-TC with restriction endonucleases Eco RI and Xba I and containing the tetracycline resistance gene of pBR322 was end-blunted by the treatment with the Klenow fragment. In the above-constructed pHIS714K2, the restriction endonuclease Nhe I site of IS714 located in the position which does not impair the transposase function was end-blunted by the treatment with the Klenow fragment. The above-mentioned fragment was ligated with this r striction endonuclease Nhe I site to transform Escherichia coli. Colonies grown in the L-agar-medium containing 25 μg/ml of Tc were obtained. The clone having inserted therein the tetracycline resistance gene fragment was selected. The plasmid contained in this clone was designated pHTN7156 as shown in Fig. 9.

On the other hand, Escherichia coli AJ12691 (WO94/25605) having plasmid p399AK9B containing an aspartoki-

nase gene which was derived from a lysine-producing mutant of Brevibacterium lactofermentum and which is desensitized to the concerted inhibition of lysine and threonine was listed as deposited at the National Institute of Bioscience and Human Technology of the Agency of Industrial Science and Technology, Ministry of Industrial Trade and Industry (1-3, Higashi 1 Chome Tsukuba-shi Ibaraki-ken 305, Japan) on April 10, 1992 under FERM P-12198, and was transferred to the deposition based on the Budapest Treaty on February 10, 1995. Deposit No. FERM BP-4999 is allotted thereto.

This p399AK9B was cleaved with restriction endonuclease Bam HI, and was self-ligated to construct pHSG399AK from which the replication origin that functions in coryneform bacterium was removed. This pHSG399AK was cleaved with Eco RI and Sph I to obtain an aspartokinase gene fragment of approximately 1.7 kb. This fragment was end-blunted by the treatment with the T4 DNA polymerase. The restriction endonuclease Bgl II site of plasmid pHTN7156 which had the artificial transposon containing the tetracycline resistance gene was blunted by the treatment with the Klenow fragment. The above-formed fragment was then inserted into this restriction endonuclease Bgl II site. In this manner, plasmid pHTN7156-C was constructed as shown in Fig. 9.

Escherichia coli AJ13131 obtained by transforming Escherichia coli with plasmid pHTN7156-C was listed as deposited at the National Institute of Bioscience and Human Technology of the Agency of Industrial Science and Technology, Ministry of Industrial Trade and Industry (1-3, Higashi 1 Chome Tsukuba-shi Ibaraki-ken 305, Japan) on June 29, 1995. Deposit No. FERM P-15014 is allotted thereto. Escherichia coli AJ13131 was transferred to the deposit based on the Budapest Treaty on May 16, 1996. Deposit No. BP-5540 is allotted thereto.

Evaluation of the number of copies in a chromosome which are formed by transposition of an artificial transposon

Brevibacterium lactofermentum AJ12036 or Brevibacterium lactofermentum AJ3445 was transformed with pHTN7156-C. The number of copies of a transposon in a chromosome which were formed by transposition of an artificial transposon into a host chromosome was evaluated. The AJ12036 strain has a wild aspartokinase gene in the chromosome, while the AJ3445 strain exhibits S-2-amylethyl-L-cysteine resistance and has an aspartokinase gene which is desensitized to concerted inhibition of lysine and threonine.

Brevibacterium lactofermentum AJ12036 was listed as deposited at the National Institute of Bioscience and Human Technology of the Agency of Industrial Science and Technology, Ministry of Industrial Trade and Industry (1-3, Higashi 1 Chome Tsukuba-shi Ibaraki-ken 305, Japan) on March 26, 1984. Deposit No. FERM P-7559 is allotted thereto. Brevibacterium lactofermentum AJ12036 was transferred to the deposit based on the Budapest Treaty on March 13, 1985. Deposit No. BP-734 is allotted thereto.

Brevibacterium lactofermentum AJ3445 was listed as deposited at the National Institute of Bioscience and Human Technology of the Agency of Industrial Science and Technology, Ministry of Industrial Trade and Industry (1-3, Higashi 1 Chome Tsukuba-shi Ibaraki-ken 305, Japan) on March 2, 1973. Deposit No. FERM P-1944 is allotted thereto. Brevibacterium lactofermentum AJ12036 was transferred to the deposit based on the Budapest Treaty on May 17, 1996. Deposit No. BP-5541 is allotted thereto.

First, the transformant was incubated overnight at 25°C in a CM2G medium containing 0.7 μg/ml of Tc, 10 g/liter of yeast extract, 10 g/liter of tryptone, 5 g/liter of glucose and 15 g/liter of NaCl. The culture was appropriately diluted with a 0.9-% NaCl solution, and was spread on the above-mentioned CM2G agar media containing from 1.5 μg/ml to 5 μg/ml of Tc in an amount of 100 μl. The resulting culture was incubated at 34°C. Some clones were selected randomly from among colonies formed, and were replicated in the CM2G agar medium containing 25 μg/ml of Km. Km-sensitive strains were then selected. Chromosomal DNAs of the Km-sensitive strains selected were produced, completely digested with restriction endonuclease Bg1 II, subjected to agarose gel electrophoresis, and blotted on a polyvinylidene difluoride (PVDF) filter. This filter was subjected to the southern hybridization using a ^{32}-P-labelled aspartokinase gene fragment (of 440 bp from the Hind III site to the Eco RI site of the gene latter half) as a probe, and the number of bands hybridized with this probe was detected. As a result, it was found that when AJ12036 was used as a host, two copies of transposon Tn7156-C were transposed in the 4 of the 10 strains analyzed, and that when AJ3445 was used as a host, two copies of transposon Tn7156-C were transposed in the 8 of the 22 strains analyzed. This proved that plural copies of the useful gene can be transduced into the chromosome at high frequency by using a tetracycline resistance gene as the selective drug resistance gene.

Evaluation of an amount of lysine produced in a strain in which an aspartokinase gene was transposed using an artificial transposon

The amount of lysine produced in the above-mentioned strain containing the transposon transposed therein was evaluated.

The strain containing the transposon was spread on the overall surface of a CM2G agar medium containing 0.7 μg/ml of Tc, and was incubated overnight at 34°C. The cells in an amount which was 1/6 of the original amount were inoculated in 20 ml of a lysine-productive medium containing 100 g/liter of glucose, 55 g/liter of ammonium sulfate, 50

ml/liter of Mamenou (Ajinomoto Co., Inc.), 1 g/liter of potassium dihydrogen phosphate, 1 g/liter of magnesium sulfate, 2 mg/liter of vitamin B1, 0.5 mg/liter of biotin, 5 mg/liter of nicotinic acid amide, 2 mg/liter of iron sulfate and 2 mg/liter of manganese sulfate (this medium was adjusted to a pH of 7.5, and then sterilized in an autoclave at 115°C for 15 minutes, after which 50 g/liter of calcium carbonate were added thereto). The culture solution was incubated in a Sakaguchi flask at 30°C for 72 hours. The content of lysine formed in the culture solution was analyzed, and the amount of lysine produced in the artificial transposon-containing strain was evaluated. Consequently, as shown in Tables 3 and 4, when AJ12036 and AJ3445 were used as parent strains, the increase in the amount of lysine produced was observed in the transposition of Tn7156-C as compared to the transposon-free strain. Further, the more the number of transposition copies (1 copy and 2 copies) of the transposon, the more the amount of lysine produced.

This proved that the amount of the amino acid produced in the strain could be increased by transducing copies of the useful gene using the tetracycline resistance gene as a selective drug resistance gene.

Table 3

Amount of lysin produced in a strain containing a transposon transposed therein using AJ12036 as a parent strain		
Strain	Number of transposition copies of Tn7156-C	Amount of lysine produced (g/liter)
AJ12306	0	0.0
Tn7156-Cint-Y1	1	12.8
Tn7156-Cint-Y2	2	18.8

Table 4

Amount of lysin produced in a strain containing a transposon transposed therein using AJ3445 as a parent strain		
Strain	Number of transposition copies of Tn7156-C	Amount of lysine produced (g/liter)
AJ3445	0	18.7
Tn7156-Cint-06	1	21.3
Tn7156-Cint-019	2	25.2

Example 5

Construction of shuttle vector pVK7

There is pAM330 which is a cryptic plasmid present in Brevibacterium lactofermentum, as described in Japanese Patent Publication No. 11,280/1989 and USP 4,788,762. This pAM330 is produced from Brevibacterium lactofermentum ATCC13869, and it can be used as a replication origin of a shuttle vector which is amplifiable in Brevibacterium.

A novel shuttle vector was constructed by combining pHSG299 (made by Takara Shuzo) which is a multi-purpose vector for E. coli with pAM330.

pAM330 was cleaved with restriction endonuclease Hind III at one site, and the cleaved surface was end-blunted with a T4 DNA polymerase. Further, pHSG299 was cleaved with restriction endonuclease Ava II at one site, and the

cleaved surface was end-blunted with a T4 DNA polymerase. The resulting fragments were ligated with each other to obtain a plasmid which was a combination of pAM330 and pHSG299. The construction of pVK7 is schematically shown in Fig. 18. pVK7 is replicable in E. coli and Brevibacterium, and imparts kanamycin resistance to a host. This vector has Pst I, Sal I, Bam HI, Kpn I, Sac I and Eco RI cloning sites each of which allows cleavage at one site and is derived from multiple cloning sites of pHSG299.

Construction of shuttle vector pVC7

Novel shuttle vector pVC7 was constructed, like pVK7, by combining pHSG399 (made by Takara Shuzo) which is a multi-purpose vector for E. coli with pAM330.

pAM330 was cleaved with restriction endonuclease Hind III at one site, and the cleaved surface was end-blunted with a T4 DNA polymerase. Further, pHSG399 was cleaved with restriction endonuclease Bsa I at one site and end-blunted with a T4 DNA polymerase. The resulting fragments were ligated with each other to obtain a plasmid which was a combination of pAM330 and pHSG399. The construction of pVC7 was schematically shown in Fig. 19. pVC7 is replicable in E. coli and Brevibacterium, and imparts kanamycin resistance to a host. This vector has Pst I, Sal I, Bam HI, Kpn I, Sac I, Eco RI, Sma I and Hind III cloning sites each of which allow cleavage at one site, among multiple cloning sites of pHSG399.

Production of a plasmid containing dapA, dapB and lysA

(1)Preparation of lysA and construction of plasmid containing lysA

A wild type strain of Brevibacterium lactofermentum ATCC 13869 was used as a chromosomal DNA donor. Chromosomal DNA was prepared from the ATCC 13869 strain in accordance with an ordinary method. A DNA fragment containing argS, lysA, and a promoter of an operon containing them was amplified from the chromosomal DNA in accordance with PCR. As for DNA primers used for amplification, synthetic DNA's of 23-mers having nucleotide sequences depicted in SEQ ID NOs: 13 and 14 in Sequence Listing respectively were used in order to amplify a region of about 3.6 kb coding for arginyl-tRNA synthase and DDC on the basis of a sequence known for Corynebacterium glutamicum (see Molecular Microbiology, 4(11), 1819-1830 (1990); Molecular and General Genetics, 212, 112-119 (1988)). Synthesis of DNA and PCR were performed by the conventional method. That is, DNA was synthesized in accordance with an ordinary method by using DNA synthesizer model 380B produced by Applied Biosystems and using the phosphoamidite method (see Tetrahedron Letters (1981), 22, 1859). The gene was amplified by PCR by using DNA Thermal Cycler Model PJ2000 produced by Takara Shuzo, and using Taq DNA polymerase in accordance with a method designated by the supplier. The sequence of the amplified DNA fragment is shown in the sequence number 15.

pHSG399 was used as a cloning vector for the amplified gene fragment of 3,579 bp. pHSG399 was digested with a restriction enzyme SmaI, which was ligated with the DNA fragment containing amplified lysA. A plasmid obtained as described above, which had lysA originating from ATCC 13869, was designated as p399LYSA.

A DNA fragment containing lysA was extracted by digesting p399LYSA with KpnI and BamHI.

This DNA fragment was ligated with pHSG299 having been digested with KpnI and BamHI. An obtained plasmid was designated as p299LYSA. The process of construction of p299LYSA is shown in Fig. 20.

p399LYSA was cleaved with restriction endonucleases Kpn I and Bam HI to extract a lysA fragment. This fragment was ligated with pVK7 cleaved with Kpn I and Bam HI. The thus-produced plasmid is designated pLYSAm (Fig. 21).

(2) Preparation of dapA and construction of plasmid containing dapA

A wild type strain of Brevibacterium lactofermentum ATCC 13869 was used as a chromosomal DNA donor. Chromosomal DNA was prepared from the ATCC 13869 strain in accordance with an ordinary method. A DNA fragment containing dapA was amplified from the chromosomal DNA in accordance with PCR. As for DNA primers used for amplification, DNA's of 20-mers having nucleotide sequences shown in SEQ ID NOs: 16 and 17 in Sequence Listing respectively were synthesized in order to amplify a region of about 1.5 kb coding for DDPS on the basis of a sequence known for Corynebacterium glutamicum (see Nucleic Acids Research, 18(21), 6421 (1990); EMBL accession No. X53993). Synthesis of DNA and PCR were performed by the conventional method. The sequence of the amplified DNA fragment is shown in the sequence number 18. pCR1000 (produced by Invitrogen, see Bio/Technology, 9, 657-663 (1991)) was used as a cloning vector for the amplified gene fragment of 1,411 bp, which was inserted into the amplified dapA fragment. Ligation of DNA was performed by using DNA ligation kit (produced by Takara Shuzo) in accordance with a designated method. Thus a plasmid was constructed, in which the dapA fragment of 1,411 bp amplified from chromosome of Brevibacterium lactofermentum was ligated with pCR1000. The plasmid obtained as described above, which had dapA originating from ATCC 13869, was designated as pCRDAPA (Fig. 22).

A transformant strain AJ13106 obtained by introducing pCRDAPA into E. coli strain has been internationally depos-

ited since May 26, 1995 under a deposition number of FERM BP-5113 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology of Ministry of Trade and Industry (postal code: 305, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) based on the Budapest Treaty.

Plasmid pCRDAPA containing dapA was digested with Kpn I and Eco RI and isolate the DNA fragment containing dapA. The fragment was ligated with pHSG399 digested with KpnI and EcoRI to obtain p399DPS (Fig. 23).

(3) Preparation of wild type and mutant lysC's and preparation of plasmids containing them

A strain of Brevibacterium lactofermentum ATCC 13869, and an L-lysine-producing mutant strain AJ3445 obtained from the ATCC 13869 strain by a mutation treatment were used as chromosomal DNA donors. The AJ3445 strain had been subjected to mutation so that lysC was changed to involve substantial desensitization from concerted inhibition by lysine and threonine (Journal of Biochemistry, 68, 701-710 (1970)).

A DNA fragment containing lysC was amplified from chromosomal DNA in accordance with the PCR method (polymerase chain reaction; see White, T. J. et al., Trends Genet., 5, 185 (1989)). As for DNA primers used for amplification, single strand DNA's of 23-mer and 21-mer having nucleotide sequences shown in SEQ ID NOs: 19 and 20 were synthesized in order to amplify a region of about 1,643 bp coding for lysC on the basis of a sequence known for Corynebacterium glutamicum (see Molecular Microbiology (1991), 5(5), 1197-1204; and Mol. Gen. Genet. (1990), 224, 317-324). The synthesis of DNA and the amplification of DNA were carried out by the conventional method. The sequence of the amplified DNA is shown in the sequnce number 21. The amplified gene fragment of 1,643 kb was confirmed by agarose gel electrophoresis. After that, the fragment excised from the gel was purified in accordance with an ordinary method, and it was digested with restriction enzymes NruI and EcoRI.

pHSG399 was used as a cloning vector for the gene fragment. pHSG399 was digested with restriction enzymes SmaI and EcoRI, and it was ligated with the amplified lysC fragment. DNA was ligated by using DNA ligation kit (produced by Takara Shuzo) in accordance with a designated method. Thus plasmids were prepared, in which the lysC fragments amplified from chromosomes of Brevibacterium lactofermentum were ligated with pHSG399 respectively. A plasmid comprising lysC from ATCC 13869 (wild type strain) was designated as p399AKY, and a plasmid comprising lysC from AJ3445 (L-lysine-producing bacterium) was designated as p399AK9 (fig. 24).

(4) Preparation of dapB and construction of plasmid containing dapB

A wild type strain of Brevibacterium lactofermentum ATCC 13869 was used as a chromosomal DNA donor. Chromosomal DNA was prepared from the ATCC 13869 strain in accordance with an ordinary method. A DNA fragment containing dapB was amplified from the chromosomal DNA in accordance with PCR. As for DNA primers used for amplification, DNA's of 23-mers having nucleotide sequences depicted in SEQ ID NOs: 22 and 23 in Sequence Listing respectively were synthesized in order to amplify a region of about 2.0 kb coding for DDPR on the basis of a sequence known for Brevibacterium lactofermentum (see Journal of Bacteriology, 157(9), 2743-2749 (1993)). Synthesis of DNA and PCR were performed by the conventional method. The sequence of the amplified DNA is shown in the sequence number 24. pCR-Script (produced by Invitrogen) was used as a cloning vector for the amplified gene fragment of 2,001 bp, which was ligated with the amplified dapB fragment. Thus a plasmid was constructed, in which the dapB fragment of 2,001 bp amplified from chromosome of Brevibacterium lactofermentum was inserted into pCR-Script. The plasmid obtained as described above, which had dapB originating from ATCC 13869, was designated as pCRDAPB (Flg. 25). A transformant strain AJ13107 obtained by introducing pCRDAPB into E. coli . strain has been internationally deposited since May 26, 1995 under a deposition number of FERM BP-5114 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology of Ministry of International Trade and Industry (postal code: 305, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) based on the Budapest Treaty.

(5) Construction of Plasmid Comprising Combination of Mutant lysC, dapA, and dapB

p399DPS was cleaved with EcoRI and SphI to form blunt ends followed by extraction of a dapA gene fragment. This fragment was ligated with the p399AK9 having been digested with SalI and blunt-ended to construct a plasmid p399CA in which mutant lysC and dapA co-existed.

The plasmid pCRDAPB comprising dapB was digested with EcoRI and blunt-ended, followed by digestion with SacI to extract a DNA fragment of 2.0 kb comprising dapB. The plasmid p399CA comprising dapA and mutant lysC was digested with SpeI and blunt-ended, which was thereafter digested with SacI and ligated with the above-extracted 2.0 kb of dapB fragment t obtain a plasmid comprising mutant lysC, dapA, and dapB. This plasmid was d signated as p399CAB (Fig. 26).

Subsequently, p399CAB was cleaved with Sac II, and the cleaved fragment was end-blunted. Then, a fragment containing dap A and dap B was extracted therefrom. Meanwhile, pLYSAm was cleaved with Bam HI, and the cleaved fragment was end-blunted. These fragments were ligated with one another to produce a plasmid which contained dapA,

dapB and lysA and which could be self-proliferated in coryneform bacteria. This plasmid is designated pABLm. The construction of pABLm is schematically shown in Fig. 21.

Transduction of the plasmid containing dapA, dapB and lysA into Brevibacterium lactofermentum Tn7156-Cint-Y2

The above-produced plasmid pABLm containing dapA, dapB and lysA was introduced into Brevibacterium lactofermentum Tn7156-Cint-Y2 by the electric pulse method [Japanese Laid-Open Patent Application (Kokai) no. 207,791/1990 by Sugimoto et al.]. The transformant was selected by the drug resistance marker and the kanamycin resistance gene of the plasmid and the tetracycline resistance gene amplified in the chromosome. Thus, the selection of the transformant was conducted in a complete culture medium containing 25 μg/ml of kanamycin and 1.5 μg/ml of tetracycline. This transformant is designated Tn7156-Cint-Y2/paBLm.

Transduction of the plasmid containing lysC, dapA, dapB and lysA into Brevibacterium lactofermentum wild strain

A DNA fragment (hereinafter referred to as "Brevi.-ori") having an ability to make a plasmid autonomously replicable in bacteria belonging to the genus Corynebacterium was introduced into p399CAB.

Brevi.-ori was prepared from a plasmid vector pHK4 containing Brevi.-ori and autonomously replicable in cells of both Escherichia coli and bacteria belonging to the genus Corynebacterium. pHK4 was digested with restriction enzymes BamHI, and cleaved edges were blunt-ended. Blunt end formation was performed by using DNA Blunting kit (produced by Takara Shuzo) in accordance with a designated method. After the blunt end formation, a phosphorylated Kpn I linker (produced by Takara Shuzo) was ligated to make modification so that the DNA fragment corresponding to the Brevi.-ori portion might be excised from pHK4 by digestion with only Kpn I. This plasmid was digested with Kpn I, and the generated Brevi.-ori DNA fragment was ligated with p399CAB having been also digested with Kpn I to prepare plasmids containing the lysC, dapA and dapB genes and autonomously replicable in bacteria belonging to the genus Corynebacterium. The plasmid was designated as pCAB. The schematic flow of constructing pCAB is shown in Fig. 26.

pHK4 was constructed by digesting pHC4 with KpnI and BamHI, extracting a Brevi.-ori fragment, and ligating it with pHSG298 having been also digested with KpnI and BamHI (see Japanese Patent Laid-open No. 5-7491). pHK4 gives kanamycin resistance to a host. Escherichia coli harboring pHK4 was designated as Escherichia coli AJ13136, and deposited on August 1, 1995 under a deposition number of FERM BP-5186 in National Institute of Bioscience and Human Technology of Agency of Industrial Science and Technology of Ministry of International Trade and Industry (postal code: 305, 1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan). (2)The plasmid p299LYSA comprising lysA was digested with KpnI and BamHI and blunt-ended, and then a lysA gene fragment was extracted. This fragment was ligated with pCAB having been digested with HpaI to construct a plasmid comprising a combination of mutant lysC, dapA, dapB, and lysA autonomously replicable in coryneform bacteria. The constructed plasmid was designated as pCABL. The process of construction of pCABL is shown in Fig. 27. It is noted that the lysA gene fragment is inserted into a HpaI site in a DNA fragment containing the dapB gene in pCABL, however, the HpaI site is located upstream from a promoter for the dapB gene (nucleotide numbers 611 to 616 in SEQ ID NO: 24), and the dapB gene is not decoupled.

The above-produced plasmid pCABL containing lysC, dapA, dapB and lysA was introduced into Brevibact rium lactofermentum wild strain AJ12036, and the selection of the transformant was conducted in a complete culture medium containing 5 μg/ml of chloramphenicol. This transformant is designated AJ12036/pCABL.

Evaluation of incubation of the above-constructed strain

Transformants AJ12036/pCABL and Tn7156-Cint-Y2/paBLm of Brevibacterium lactofermentum wild strain AJ12036 were incubated in an L-lysine-productive culture medium, and the amount of L-lysine produced therein was evaluated. The composition of the L-lysine-productive culture medium was as follows.

L-lysine-productive culture medium:

The following ingredients (in amounts per liter) except calcium carbonate were dissolved, and the solution was adjusted to a pH of 8.0 with KOH. The resulting solution was sterilized at 115°C for 15 minutes, and 50 g of calcium carbonate which had been separately dry-sterilized were added thereto.

glucose	100 g
$(NH_4)_2SO_4$	55 g
KH_2PO_4	1 g
$MgSO_4 \cdot 7H_2O$	1 g
biotin	500 µg
Thiamine	2000 µg
$FeSO_4 \cdot 7H_2O$	0.01 g
$MnSO_4 \cdot 7H_2O$	0.01 g
nicotinamide	5 mg
protein hydrolyzate (Mamenou)	30 ml
calcium carbonate	50 g

The parent strain and the transformant were inoculated in the culture medium having the above-mentioned composition, and were incubated at 31.5°C while being shaken reciprocally. The amount of L-lysine produced after 72 hours of the incubation, the growth (OD_{562}) and the stability given when the incubation was completed are shown in Table 5. The growth was evaluated by diluting the solution to 101 times and measuring OD at 562 nm. Further, with respect to the stability, the culture solution in the completion of the incubation was grown in a complete culture medium after th dilution, and the colonies formed were put on a drug-containing plate, and the stability was indicated as growth rate of the colonies formed on the drug-containing plate.

Table 5

Strain/plasmid	Growth	Amount of L-lysine produced (g/liter)	Stability (%)
AJ12036	0.700	0.0	-
AJ12036/pCABL	0.590	28.1	90
Tn7156-Cint-Y2/paBLm	0.608	28.5	100

As shown in Table 5, the amount of lysine produced was improved when using the strain in which lysC was increased in the plasmid as well as when using the strain in which lysC was increased in the chromosome. Further, the stability of AJ123036/pCABL was 90%, while that of Tn7156-Cint-Y2/paBLm was 100%.

Example 6

Construction of plasmid pHTN7150

Since the above-constructed artificial transposon Tn7145 carrying a kanamycin resistance gene did not have a suitable site for a dihydrodipicolinic acid synthase to be inserted, a new plasmid pHTN7150 into which a new insert site was introduced was constructed as follows.

A kanamycin resistance gene was cut out from plasmid vector pUC4K (produced by Pharmacia Biotech) with a restriction enzyme Pst I and made blunt-ended. The fragment containing the kanamycin resistance gene was inserted into the Sma I site of pHY300PLK (produced by Takara Shuzo) to construct pHY300-KM. Then pHY300-KM was digested with restriction enzymes Eco RI and Xba I to cut out a fragment containing a kanamycin resistance gene. This fragment was made blunt-ended and inserted into the blunt-ended Nhe I site of IS714 on the plasmid pHIS714 to construct plasmid pHTN 7150. The artificial transposon Tn7150 on pHTN7150 has a kanamycin resistance gene as a marker gene and a Bgl II site which can be used as a gene cloning site.

Combination of pHTN7150 and dapA gene of Brevibacterium lactofermentum

A gene encoding a dihydrodipicolinic acid synthase which is a lysine biosynthetase gene was inserted int artificial transposon pHTN7150 containing a kanamycin resistance gene in the following manner.

After plasmid p399DPS containing dapA was cleaved with Eco RI, the resulting fragment was then end-blunted through the treatment with a T4 DNA polymerase, and a phosphorylated Bam HI linker (made by Takara Shuzo) was bound therewith to modify the fragment such that the dapA gene could be cut out with Bam HI alone. This plasmid is designated p399DPS2. A dapA fragment of 1.4 kb formed by cleaving this plasmid with Bam HI was combined with pHTN7150 cleaved with Bgl II that gives the same cohesive end as Bam HI. The thus-constructed plasmid is designated pHTN7150A. The construction of pHTN7150A is schematically shown in Fig. 28.

Transposition of the artificial transposon Tn7150A into a chromosome of Brevibacterium lactofermentum

A strain formed by transposing the artificial transposon Tn7150A containing dapA into Brevibacterium lactofermentum AJ12036 strain was obtained using pHTN7150A in the following manner.

The AJ12036 strain was transformed with pHTN7150A. The resulting transformant was incubated overnight at 25°C in a CM2S liquid medium containing 25 μg/ml of kanamycin, 10 g/liter of yeast extract, 10 g/liter of tryptone, 5 g/liter of sucrose and 15 g/liter of NaCl. The culture was diluted approximately with a 0.9-% NaCl solution. The dilute was spread on the above-mentioned CM2S agar medium containing 25 μg/ml, and was incubated at 34°C. Chloramphenicol-sensitive strains were selected from among colonies formed, and some of these strains were randomly selected. Chromosomal DNAs were prepared therefrom, and subjected to the southern hybridization using the dapA fragment as a probe to identify the transposition of the artificial transposon. The above-obtained strain having transposed therein the artificial transposon is designated AJ12036::A.

Construction of pCBLmc and production of a strain

A plasmid containing variant lysC, dapB and lysA was constructed using pVC7, a shuttle vector of pAM330 and pHSG399 in the following manner.

After pCRDAPB containing dapB was treated with Sac I, the resulting fragment was end-blunted through the treatment with a T4 DNA polymerase to construct a plasmid combined with a phosphorylated Pst I linker (made by Takara Shuzo). The thus-obtained plasmid is designated pCRDAPB2. This plasmid was cleaved with Bam HI and Pst I, and the resulting dapB fragment of 2.0 kb was inserted into pVC7 cleaved with Bam HI and Pst I. This plasmid is designated pBmc.

pAK9 containing lysC was cleaved with Bam HI and Eco RI, and the resulting lysC fragment of 1.6 kb was connected to pBmC cleaved also with Bam HI and Eco RI to construct a plasmid containing dapB and lysC. This plasmid is designated pBCmc.

After p399LYSA containing lysA was cleaved with Eco RI, the resulting fragment was end-blunted through the treatment with a T4 DNA polymerase, and was combined with a phosphorylated Kpn I linker to modify it such that lysA was cleaved with Kpn I. This plasmid is designated p399LYSA2. p399LYSA2 was cleaved with KpnI. The resulting lysA fragment of 3.6 kb was ligated with pBCmc having been digested with Eco RI, end-blunted through the treatment with a T4 DNA polymerase, and combined with the phosphorylated Kpn I linker. The thus-obtained plasmid is designated pCBLmc. This plasmid was self-replicable in E. coli and coryneform bacteria, imparted chloramphenicol resistance to a host, and contained mutant lysC, dapB and lysA. The construction of pCBLmc is schematically shown in Fig. 29.

The above-constructed pCBLm was introduced into the AJ12036::A strain in which the artificial transposon Tn7150A had been transposed into the chromosome by the electric pulse method [Japanese Laid-Open Patent Application (Kokai) No. 207,791/1990 by Sugimoto et al.]. The selection of the transformants was conducted in the above-mentioned CM2S medium containing 5 μg/ml of chloramphenicol and 25 μg/ml of kanamycin. The thus-constructed stain is designated AJ12036::A/pCBLmc.

Evaluation of incubation of the strains constructed

The parent strain and the transformants AJ12036/pCABL and AJ12036::A/pCBLmc were incubated in a L-lysine-productive culture medium, and the amount of lysine produced was evaluated. The result was shown in Table 6.

Table 6

Strain/plasmid	Growth	Amount of lysine produced (g/liter)	Stability (%)
AJ12036	0.700	0.0	-
AJ12036/pCABL	0.590	28.1	90
AJ12036::A/pCBLmc	0.595	28.7	100

As is apparent from Table 6, the amount of lysin produced was improved in the strain in which dapA was increased in the chromosome as was seen and in the strain in which lysC was increased in the plasmid. Further, the stability of AJ12036/pCABL was 90%, while that of AJ12036::A/pCBLm was 100%.

Example 7

Construction of an artificial transposon containing no transposase in a transposon unit Construction of a transposase expression plasmid using an E. coli Trc promoter or the like

Plasmid pHIS714 was cleaved with restriction endonucleases Nhe I and Xba I to obtain a fragment containing a gene encoding a transposase from which a 5'-side inverted repeat (IR) of IS714 was deleted. This DNA fragment was introduced into an Xba I site of plasmid vector pUC19 to construct plasmid TnpL/pUC19.

Further, TnpL/pUC19 was cleaved with restriction endonucleases Mro I and Xba I to delete a sequence including a termination codon of IS714 and a 3'-side inverted repeat (IR). A synthetic double-stranded DNA having the following sequence was inserted into the above-cleaved portion through ligation.

5'-CCGGACAGCTCACCCACAAAATCAATGCACTCTAAAAAGGTACCT -3'

3'- TGTCGAGTGGGTGTTTTAGTTACGTGAGATTTTTCCATGGAGATC-5'

(Sequence number 25 and 26)

In this manner, plasmid ORFL/pUC19 was constructed in which IR present in the transposase 3'-sid of TnpL/pUC19 was deleted.

Subsequently, this ORFL/pUC19 was cleaved with restriction endonucleases Sma I and Xba I to obtain a gene fragment of approximately 1.5 kb containing the transposase. This transposase gene fragment was inserted into a portion of plasmid vector pHY300PLK (made by Takara Shuzo) obtained by removing a sequence between Sma I and Xba I sites thereof, and was then cut out with restriction endonucleases Eco RI and Kpn I. This Eco RI-Kpn I transposase gene fragment was end-blunted with a T4 DNA polymerase. Meanwhile, plasmid vector pHSG398 (made by Takara Shuzo) was partially digested with restriction endonuclease Pvu II to delete a fragment of approximately 0.3 kb containing a multicloning site. The above-obtained transposase gene fragment was inserted into the digested portion of plasmid vector pHSG398 to construct plasmid pORF1 as shown in Fig. 10.

On the other hand, the Nhe I - Xba I cleavage fragment of plasmid pHIS714 which had been obtained earlier was end-blunted, and introduced into the end-blunted Pst I site of plasmid vector pUC19 to construct plasmid Tnp (Pst)/pUC19.

The transposase gene of this Tnp(Pst)/pUC19 was subjected to the partial base substitution using a U. S. E. Mutagenesis Kit (made by Pharmacia Biotech). The base substituted was G which was the 288th base in the sequence of IS714. This base G was replaced with C. This was a change from GTG to GTC, and it was not a change of an amino-acid level. This base-substituted plasmid is designated Tnp(Pst)M/pUC19.

The sequence between restriction endonuclease Sma I and Nae I sites present in the transposase first half gene was deleted from pORF1. The transposase first half gene fragment (including the change GTG → GTC) obtained by cleaving Tnp(Pst)M/pUC19 with restriction endonucleases Sma I and Nae I was inserted into the above-deleted portion through ligation to construct pORF2.

The sequence between the Sma I and Xba I sites was deleted from pORF2, and the resulting fragment was end-blunted. A DNA fragment containing a tryptophan operon attenuator was obtained by cleaving pBSF2-SD7 with restriction endonucleases Nae I and Hind III, and was then end-blunted. The former fragment was ligated with the latter fragment. The thus-constructed plasmid is designated pORF3.

E. coli HB101 transformed with plasmid pBSF2-SD7 (AJ12448) was listed as deposited at the National Institute of Bioscience and Human Technology of the Agency of Industrial Science and Technology, Ministry of International Trade and Industry, (1-3, Higashi 1 Chome Tsukuba-shi Ibaraki-ken 305, Japan) under deposit No. FERM P-10758 on June 1, 1989. The strain was transferred to the deposit based on the Budapest Treaty on February 19, 1992. Deposit No. BP-

3753 is allotted thereto.

pORF3 was cleaved with restriction endonucleases Sal I and Bpu II02I to delete the transposase first half gene fragment. The transposase first half gene fragment obtained by cleaving Tnp(Pst)/pUC19 with restriction endonucleases Sal I and Blu II02I was inserted into the above-deleted portion by ligation to construct pORF4 as shown in Fig. 11.

TnpL/pUC19 was cleaved with Sac I, and was then digested with BAL 31 nuclease at 30°C for 20 minutes to delete a sequence near the initiation codon of the transposase gene from the upstream side. After the ends which underwent the deletion were blunted, the transposase gene fragment was cut out using the Sph I site, and was inserted into a site of pHSG398 which was cleaved with Sma I and Sph I. The thus-constructed plasmid is designated delTnp5/398.

This delTnp5/398 was cleaved with restriction endonucleases Knp I and Hind III, and the resulting transposase first half gene fragment was end-blunted. Then, plasmid vector pKK233-2 (made by Pharmacia Biotech) was cleaved with Nco I and Hind III, and was end-blunted. The former fragment was ligated with the latter fragment though ligation to construct pTrc-ORF.

pTrc-ORF was cleaved with Ssp I and Bpu 1102I to form a fragment containing Trc promoter and the transposase first half gene. pORF3 was cleaved with Xba I, end-blunted, and further cleaved with Bpu II02I to delete the transposase first half gene fragment. The above-formed fragment was inserted into this deleted portion of pORF3 to construct pORF7 as shown in Fig. 12.

The transposase first half gene fragment obtained by cleaving delTnp5/398 with restriction endonucleases Kpn I and Hind III was cloned between the KpnI and Hind III sites of plasmid vector pUC18. The portion between the Bsm I and Nae I sites of this plasmid was deleted, and the fragment was ligated with the transposase first half gene fragment (G → C substitution type) obtained by cleaving Tnp(Pst)M/pUC19 with restriction endonucleases Bsm I and Nae I to construct delTnp5M/18.

This delTnp5M/18 was cleaved with Kpn I and Hind III, and the resulting transposase first half gene fragment was end-blunted. pKK233-2 was cleaved with Nco I and Hind III, and the resulting fragment was end-blunted. These fragments were ligated with each other to construct pTrc-TnpM.

pORF8 was constructed from pTrc-TnpM by the method of constructing pORF7 from pTrc-Tnp (Fig. 13).

Construction of a plasmid for introduction of a coryneform bacterium containing an artificial transposon unit and a transposase expression system outside this unit

Plasmids were constructed using the above-mentioned plasmids pORF3, pORF4, pORF7 and pORF8. The construction of pORF41 from pORF3 is described below.

First, pHIS714 was cleaved with Nhe I and Sac II to delete the major part of the transposase gene. A double-stranded synthetic DNA having the following sequence was inserted into the above-deleted portion to construct pHTN7160.

5'-CTAGCTCGAGATATCAGATCTACTAGTCGACCGC-3' Sequence number 27
3'- GAGCTCTATAGTCTAGATGATCAGCTGG -5' Sequence number 28

pHTN7160 was cleaved with restriction endonuclerase Kpn I, end-blunted, and then cleaved again with Bgl I to obtain a fragment containing inverted repeats (IR) on both sides of IS714 and a temperature-sensitive replication origin that functions within a coryneform bacterium.

pORF3 was cleaved with restriction endonuclease Ear I, end-blunted, and then cleaved again with Bgl I. The above-mentioned fragment of pHTN7160 was inserted therein to construct pORF41-pre.

Then, pORF41-pre was cleaved with Eco RV. An Eco RI-Ava I fragment which contained the Tc resistance gene of pBR322 and was end-blunted was inserted into the Eco RV-cleaved fragment to construct pORF41 as shown in Fig. 14.

The above-mentioned method was repeated to construct pORF31 from pORF4 through pORF31-pre, pORF71 from pORF7 through pORF71-pre, and pORF81 from pORF8 through pORF81-pre, respectively.

E. coli AJ13208 harboring plasmid pORF81 was listed as deposited at the National Institute of Bioscience and Human Technology of the Agency of Industrial Science and Technology, Ministry of International Trade and Industry, (1-3, Higashi 1 Chome Tsukuba-shi Ibaraki-ken 305, Japan) based on the Budapest Treaty on June 3, 1996. Deposit No. BP-5557 is allotted thereto.

pORF3 was cleaved with Xba I and Ear I, end-blunted, and self-ligated to construct pORFC0 containing no transposase gene (Fig. 15).

pORFC2 composed only of a transposon unit (containing no transposase gene) was constructed from pORFC0 through pORFC2-pre in the same manner as in constructing pORF41 form pORF3.

These finally constructed plasmids had the structural gene of the transposase, the Cm resistance gene, the replication origin that functions within E. coli, the temp rature-sensitive replication origin that functions within a coryneform bacterium and the Tc resistance gene held between IRs of IS714, provided pORFC2 had no structural gene of the transposase.

The unit containing IRs on both ends of IS714 and the Tc resistance gene is designated transposon unit Tn7162.

Evaluation of the number of copies of the transposon unit having the Tc resistance gene in the chromosome which was formed by transposition of the transposon unit

The test of transposition was conducted using pORF31, pORF41, pORF81 and pORFC2 of the above-constructed plasmids. The unit considered to be transposed was transposon unit Tn7162.

Brevibacterium lactofermentum AJ12036 was transformed with each of the above-mentioned plasmids, and the number of copies of transposon unit Tn7162 in the host chromosome which were formed by the transposition of transposon unit Tn7162 into the host chromosome was evaluated. That is, the transformant was incubated overnight at 25°C in the above-mentioned CM2G liquid medium containing 5 μg/ml of Cm, and was appropriately diluted with a 0.9-% NaCl solution. The dilute was spread on the above-mentioned CM2G agar medium containing from 1.5 μg/ml to 4 μg/ml of Tc in an amount of 100 μl, and was incubated at 34°C. Cm-sensitive clones were selected from among the colonies formed, and were incubated at 34°C. Some of the clones were randomly selected from among the colonies formed. Chromosomal DNAs were produced therefrom, completely digested with restriction endonuclease Pvu II, subjected to agarose gel electrophoresis, and blotted on a nitrocellulose (or nylon or PVDF) filter. This filter was subjected to the southern hybridization using, as a probe, a Tc resistance gene fragment labelled with ^{32}-P or with an ECL direct labelling system (made by Amersham), and the number of bands hybridized with the probe was detected.

Consequently, it was found, as shown in Table 7, that a large number of copies of transposon unit Tn7162 having the Tc resistance marker gene were transposed at some frequency.

This proved that the expression-type transposase gene functioned either outside the transposon unit in the plasmid (pORF31, 41 and 81) or in the transposase inherently present in the chromosome (pORFC2).

Table 7

Plasmid	Selective Tc concentration (μg/ml)	Number of copies of Tc resistance gene
pORFC2	1.5	>8
	2.0	>12
pORF31	2.0	>7
pORF41	1.5	>11
pORF81	1.5	3
		4
		10
		11
	2.0	3
		4
		4
	4.0	5

Example 8

Construction of a plasmid for coryneform bacteria containing a transposase expression system alone and transposition of a transposon unit on a chromosome

Construction of a plasmid for coryneform bacteria containing a transposase expression system alone

Plasmid pHIS714K1 was cleaved with EcoO 109I and Mro I to delete IS714, and was then self-ligated to construct pHIS714Kdel.

Meanwhile, pORF3 was cleaved with r striction endonuclease Ear I, end-blunted, and cleaved again with Bgl I. pHIS714Kdel was cleaved with restriction endonuclease Kpn I, end-blunted, and then cleaved again with Bgl I to form a fragment which contained a temperature-sensitive replication origin and which functioned within coryneform bacteria. The thus-formed fragments are ligated with each other to construct pORF40 as shown in Fig. 17.

This method was repeated to construct pORF30 from pORF4, pORF70 from pORF 7, pORF80 from pORF 8 and pORFC1 from pORFC0 respectively.

Evaluation of the number of copies of the transposon unit having the Tc resistance gene in the chromosome which were formed by transposition of the transposon unit

The test of transposition was conducted using pORF80 and pORFC1 of the above-constructed plasmids. The unit considered to be transposed was transposon unit Tn7162.

In Example 7, it was demonstrated that Brevibacterium lactofermentum AJ12036 was transformed with the plasmid containing transposon unit Tn7162, and a large number of copies of Tn7162 were transposed into the host chromosome. It was tested through the southern hybridization analysis of the chromosomal DNA whether Tn7162 in the chromosome was further transposed or replicated when the above-constructed plasmids pORF80 and pORFC1 were further transduced into one copy of the chromosome transposition strain obtained here as a host to increase the transposase activity. Then, the number of copies was evaluated.

That is, the transformant was incubated overnight at 25°C in the above-mentioned CM2G liquid medium containing 5 μg/ml of Cm, and then appropriately diluted with a 0.9-% NaCl solution. The dilute was spread on the above-mentioned CM2G agar medium containing from 6 μg/ml to 20 μg/ml of Tc in an amount of 100 μl, and was incubated at 34°C. Cm-sensitive clones were selected from among colonies formed.

Some clones were randomly selected from among these Cm-sensitive clones. Chromosomal DNAs were prepared therefrom, completely digested with restriction endonuclease Pvu II, subjected to agarose gel electrophoresis, and blotted on a nitrocellulose (or nylon or PVDF) filter. This filter was subjected to the southern hybridization using, as a probe, a Tc resistance gene fragment labelled with ^{32}-P or with an ECL direct labelling system (made by Amersham), and the number of bands hybridized with this probe was detected.

As a result, a large number of copies of transposon unit Tn7162 having the Tc resistance marker gene were transposed and replicated at some frequency.

Sequence Table

(1) INFORMATION FOR SEQ ID NO:1:

1 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1453 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Brevibacterium lactofermentum

(B) STRAIN: AJ12036

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 130..1440

(ix) FEATURE:

(A) NAME/KEY: repeat_region

(B) LOCATION: 1..15

(ix) FEATURE:

(A) NAME/KEY: repeat_region

(B) LOCATION: 1439..1453

(ix) FEATURE:

(A) NAME/KEY: -35_signal

(B) LOCATION: 71..76

(ix) FEATURE:

(A) NAME/KEY: -10_signal

(B) LOCATION: 92..97

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGCCCTTCCG GTTTTGGGGT ACATCACAGA ACCTGGGCTA GCGGTGTAGA CCCGAAAATA 60

AACGAGCCTT TTGTCAGGGT TAAGGTTTAG GTATCTAAGC TAACCAAACA CCAACAAAAG 120

GCTCTACCC ATG AAG TCT ACC GGC AAC ATC ATC GCT GAC ACC ATC TGC 168

Met Lys Ser Thr Gly Asn Ile Ile Ala Asp Thr Ile Cys

1 5 10

CGC ACT GCG GAA CTA GGA CTC ACC ATC ACC GGC GCT TCC GAT GCA GGT 216

Arg Thr Ala Glu Leu Gly Leu Thr Ile Thr Gly Ala Ser Asp Ala Gly

15 20 25

GAT TAC ACC CTG ATC GAA GCA GAC GCA CTC GAC TAT ACC TCC ACC TGC 264

Asp Tyr Thr Leu Ile Glu Ala Asp Ala Leu Asp Tyr Thr Ser Thr Cys

30 35 40 45

CCA GAA TGC TTC CAA CCT GGG GTG TTT CGT CAT CAC ACC CAC CGG ATG 312

Pro Glu Cys Phe Gln Pro Gly Val Phe Arg His His Thr His Arg Met

50 55 60

CTC ATT GAT TTA CCC ATC GTC GGG TTT CCC ACC AAA CTG TTT ATC CGT 360

Leu Ile Asp Leu Pro Ile Val Gly Phe Pro Thr Lys Leu Phe Ile Arg

65 70 75

CTA CCT CGC TAC CGC TGC ACC AAC CCG ACA TGT AAG CAA AAG TAT TTC 408
Leu Pro Arg Tyr Arg Cys Thr Asn Pro Thr Cys Lys Gln Lys Tyr Phe
80 85 90

CAA GCA GAA CTA AGC TGC GCT GAC CAC GGT AAA AAG GTC ACC CAC CGG 456
Gln Ala Glu Leu Ser Cys Ala Asp His Gly Lys Lys Val Thr His Arg
95 100 105

GTC ACC CGC TGG ATT TTG CAA CGC CTT GCT ATT GAC CGG ATG AGT GTT 504
Val Thr Arg Trp Ile Leu Gln Arg Leu Ala Ile Asp Arg Met Ser Val
110 115 120 125

CAC GCA ACT GCG AAA GCA CTT GGG CTA GGG TGG GAT TTA ACC TGC CAA 552
His Ala Thr Ala Lys Ala Leu Gly Leu Gly Trp Asp Leu Thr Cys Gln
130 135 140

CTA GCC CTC GAT ATG TGC CGT GAG CTG GTC TAT AAC GAT CCT CAC CAT 600
Leu Ala Leu Asp Met Cys Arg Glu Leu Val Tyr Asn Asp Pro His His

145 150 155

CTT GAT GGA GTG TAT GTC ATT GGG GTG GAT GAG CAT AAG TGG TCA CAT 648

Leu Asp Gly Val Tyr Val Ile Gly Val Asp Glu His Lys Trp Ser His

160 165 170

AAT AGG GCT AAG CAT GGT GAT GGG TTT GTC ACC GTG ATT GTC GAT ATG 696

Asn Arg Ala Lys His Gly Asp Gly Phe Val Thr Val Ile Val Asp Met

175 180 185

ACC GGG CAT CGG TAT GAC TCA CGG TGT CCT GCC CGG TTA TTA GAT GTC 744

Thr Gly His Arg Tyr Asp Ser Arg Cys Pro Ala Arg Leu Leu Asp Val

190 195 200 205

GTC CCA GGT CGT AGT GCT GAT GCT TTA CGG TCC TGG CTT GGC TCC CGC 792

Val Pro Gly Arg Ser Ala Asp Ala Leu Arg Ser Trp Leu Gly Ser Arg

210 215 220

GGT GAA CAG TTC CGC AAT CAG ATA CGG ATC GTG TCC ATG GAT GGA TTC 840

Gly Glu Gln Phe Arg Asn Gln Ile Arg Ile Val Ser Met Asp Gly Phe

225 230 235

CAA GGC TAC GCC ACA GCA AGT AAA GAA CTC ATT CCT TCT GCT CGT CGC 888
Gln Gly Tyr Ala Thr Ala Ser Lys Glu Leu Ile Pro Ser Ala Arg Arg

240 245 250

GTG ATG GAT CCA TTC CAT GTT GTG CGG CTT GCT GGT GAC AAG CTC ACC 936
Val Met Asp Pro Phe His Val Val Arg Leu Ala Gly Asp Lys Leu Thr

255 260 265

GCC TGC CGG CAA CGC CTC CAG CGG GAG AAA TAC CAG CGT CGT GGT TTA 984
Ala Cys Arg Gln Arg Leu Gln Arg Glu Lys Tyr Gln Arg Arg Gly Leu

270 275 280 285

AGC CAG GAT CCG TTG TAT AAA AAC CGG AAG ACC TTG TTG ACC ACG CAC 1032
Ser Gln Asp Pro Leu Tyr Lys Asn Arg Lys Thr Leu Leu Thr Thr His

290 295 300

AAG TGG TTG AGT CCT CGT CAG CAA GAA AGC TTG GAG CAG TTG TGG GCG 1080
Lys Trp Leu Ser Pro Arg Gln Gln Glu Ser Leu Glu Gln Leu Trp Ala

305 310 315

TAT GAC AAA GAC TAC GGG GTG TTA AAG CTT GCG TGG CTT GCG TAT CAG 1128
Tyr Asp Lys Asp Tyr Gly Val Leu Lys Leu Ala Trp Leu Ala Tyr Gln
320 325 330

GCG ATT ATT GAT TGT TAT CAG ATG GGT AAT AAG CGT GAA GCG AAG AAG 1176
Ala Ile Ile Asp Cys Tyr Gln Met Gly Asn Lys Arg Glu Ala Lys Lys
335 340 345

AAA ATG CGG ACC ATT ATT GAT CAG CTT CGG GTG TTG AAG GGG CCG AAT 1224
Lys Met Arg Thr Ile Ile Asp Gln Leu Arg Val Leu Lys Gly Pro Asn
350 355 360 365

AAG GAA CTC GCG CAG TTG GGT CGT AGT TTG TTT AAA CGA CTT GGT GAT 1272
Lys Glu Leu Ala Gln Leu Gly Arg Ser Leu Phe Lys Arg Leu Gly Asp
370 375 380

GTG TTG GCG TAT TTC GAC GTA GGA GTC TCC AAC GGA CCA GTC GAA GCC 1320
Val Leu Ala Tyr Phe Asp Val Gly Val Ser Asn Gly Pro Val Glu Ala

385 390 395

ATC AAT GGA CGC CTA GAA CAC CTC CGC GGA ATC GCG CTT GGA TTC CGC 1368
Ile Asn Gly Arg Leu Glu His Leu Arg Gly Ile Ala Leu Gly Phe Arg
400 405 410

AAC CTC ACC CAC TAC ATC CTT CGA TGC CTC ATC CAC TCC GGA CAG CTC 1416
Asn Leu Thr His Tyr Ile Leu Arg Cys Leu Ile His Ser Gly Gln Leu
415 420 425

ACC CAC AAA ATC AAT GCA CTC TAA AAACGGAAGA GCC 1453
Thr His Lys Ile Asn Ala Leu *
430 435

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 437 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Lys Ser Thr Gly Asn Ile Ile Ala Asp Thr Ile Cys Arg Thr Ala
1 5 10 15

Glu Leu Gly Leu Thr Ile Thr Gly Ala Ser Asp Ala Gly Asp Tyr Thr
20 25 30

Leu Ile Glu Ala Asp Ala Leu Asp Tyr Thr Ser Thr Cys Pro Glu Cys
35 40 45

Phe Gln Pro Gly Val Phe Arg His His Thr His Arg Met Leu Ile Asp
50 55 60

Leu Pro Ile Val Gly Phe Pro Thr Lys Leu Phe Ile Arg Leu Pro Arg
65 70 75 80

Tyr Arg Cys Thr Asn Pro Thr Cys Lys Gln Lys Tyr Phe Gln Ala Glu
85 90 95

Leu Ser Cys Ala Asp His Gly Lys Lys Val Thr His Arg Val Thr Arg
100 105 110

Trp Ile Leu Gln Arg Leu Ala Ile Asp Arg Met Ser Val His Ala Thr
115 120 125

Ala Lys Ala Leu Gly Leu Gly Trp Asp Leu Thr Cys Gln Leu Ala Leu
130 135 140

Asp Met Cys Arg Glu Leu Val Tyr Asn Asp Pro His His Leu Asp Gly
145 150 155 160

Val Tyr Val Ile Gly Val Asp Glu His Lys Trp Ser His Asn Arg Ala
165 170 175

Lys His Gly Asp Gly Phe Val Thr Val Ile Val Asp Met Thr Gly His
180 185 190

Arg Tyr Asp Ser Arg Cys Pro Ala Arg Leu Leu Asp Val Val Pro Gly
195 200 205

Arg Ser Ala Asp Ala Leu Arg Ser Trp Leu Gly Ser Arg Gly Glu Gln
210 215 220

Phe Arg Asn Gln Ile Arg Ile Val Ser Met Asp Gly Phe Gln Gly Tyr
225 230 235 240

Ala Thr Ala Ser Lys Glu Leu Ile Pro Ser Ala Arg Arg Val Met Asp
245 250 255

Pro Phe His Val Val Arg Leu Ala Gly Asp Lys Leu Thr Ala Cys Arg
260 265 270

Gln Arg Leu Gln Arg Glu Lys Tyr Gln Arg Arg Gly Leu Ser Gln Asp
275 280 285

Pro Leu Tyr Lys Asn Arg Lys Thr Leu Leu Thr Thr His Lys Trp Leu
290 295 300

Ser Pro Arg Gln Gln Glu Ser Leu Glu Gln Leu Trp Ala Tyr Asp Lys
305 310 315 320

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Asp Tyr Gly Val Leu Lys Leu Ala Trp Leu Ala Tyr Gln Ala Ile Ile
                325                 330                 335

Asp Cys Tyr Gln Met Gly Asn Lys Arg Glu Ala Lys Lys Lys Met Arg
            340                 345                 350

Thr Ile Ile Asp Gln Leu Arg Val Leu Lys Gly Pro Asn Lys Glu Leu
        355                 360                 365

Ala Gln Leu Gly Arg Ser Leu Phe Lys Arg Leu Gly Asp Val Leu Ala
    370                 375                 380

Tyr Phe Asp Val Gly Val Ser Asn Gly Pro Val Glu Ala Ile Asn Gly
385                 390                 395                 400

Arg Leu Glu His Leu Arg Gly Ile Ala Leu Gly Phe Arg Asn Leu Thr
                405                 410                 415

His Tyr Ile Leu Arg Cys Leu Ile His Ser Gly Gln Leu Thr His Lys
            420                 425                 430
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Ile Asn Ala Leu *

435

(3) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Brevibacterium lactofermentum

(B) STRAIN: AJ12036

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGCCCTTCCG GTTTT 15

(4) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Brevibacterium lactofermentum

(B) STRAIN: AJ12036

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGCTCTTCCG TTTTT 15

(5) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1453 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Brevibacterium lactofermentum

(B) STRAIN: AJ12036

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 130..1440

(ix) FEATURE:

(A) NAME/KEY: repeat_region

(B) LOCATION: 1..15

(ix) FEATURE:

(A) NAME/KEY: repeat_region

(B) LOCATION: 1439..1453

(ix) FEATURE:

(A) NAME/KEY: -35_signal

(B) LOCATION: 71..76

(ix) FEATURE:

(A) NAME/KEY: -10_signal

(B) LOCATION: 92..97

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGCCCTTCCG GTTTTGGGGT ACATCACAGA ACCTGGGCTA GCGGTGTAGA CCCGAAAATA 60

AACGAGCCTT TTGTCAGGGT TAAGGTTTAG GTATCTAAGC TAACCAAACA CCAACAAAAG 120

GCTCTACCC ATG AAG TCT ACC GGC AAC ATC ATC GCT GAC ACC ATC TGC 168
Met Lys Ser Thr Gly Asn Ile Ile Ala Asp Thr Ile Cys
1 5 10

CGC ACT GCG GAA CTA GGA CTC ACC ATC ACC GGC GCT TCC GAT GCA GGT 216
Arg Thr Ala Glu Leu Gly Leu Thr Ile Thr Gly Ala Ser Asp Ala Gly
15 20 25

GAT TAC ACC CTG ATC GAA GCA GAC GCA CTC GAC TAT ACC TCC ACC TGC 264
Asp Tyr Thr Leu Ile Glu Ala Asp Ala Leu Asp Tyr Thr Ser Thr Cys
30 35 40 45

CCA GAA TGC TTC CAA CCT GGG GTG TTT CGT CAT CAC ACC CAC CGG ATG 312
Pro Glu Cys Phe Gln Pro Gly Val Phe Arg His His Thr His Arg Met
50 55 60

CTC ATT GAT TTA CCC ATC GTC GGG TTT CCC ACC AAA CTG TTT ATC CGT 360

Leu Ile Asp Leu Pro Ile Val Gly Phe Pro Thr Lys Leu Phe Ile Arg

65 70 75

CTA CCT CGC TAC CGC TGC ACC AAC CCG ACA TGT AAG CAA AAG TAT TTC 408

Leu Pro Arg Tyr Arg Cys Thr Asn Pro Thr Cys Lys Gln Lys Tyr Phe

80 85 90

CAA GCA GAA CTA AGC TGC GCT GAC CAC GGT AAA AAG GTC ACC CAC CGG 456

Gln Ala Glu Leu Ser Cys Ala Asp His Gly Lys Lys Val Thr His Arg

95 100 105

GTC ACC CGC TGG ATT TTG CAA CGC CTT GCT ATT GAC CGG ATG AGT GTT 504

Val Thr Arg Trp Ile Leu Gln Arg Leu Ala Ile Asp Arg Met Ser Val

110 115 120 125

CAC GCA ACT GCG AAA GCA CTT GGG CTA GGG TGG GAT TTA ACC TGC CAA 552

His Ala Thr Ala Lys Ala Leu Gly Leu Gly Trp Asp Leu Thr Cys Gln

130 135 140

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CTA GCC CTC GAT ATG TGC CGT GAG CTG GTC TAT AAC GAT CCT CAC CAT        600
Leu Ala Leu Asp Met Cys Arg Glu Leu Val Tyr Asn Asp Pro His His
                145                 150                 155

CTT GAT GGA GTG TAT GTC ATT GGG GTG GAT GAG CAT AAG TGG TCA CAT        648
Leu Asp Gly Val Tyr Val Ile Gly Val Asp Glu His Lys Trp Ser His
            160                 165                 170

AAT AGG GCT AAG CAT GGT GAT GGG TTT GTC ACC GTG ATT GTC GAT ATG        696
Asn Arg Ala Lys His Gly Asp Gly Phe Val Thr Val Ile Val Asp Met
        175                 180                 185

ACC GGG CAT CGG TAT GAC TCA CGG TGT CCT GCC CGG TTA TTA GAT GTC        744
Thr Gly His Arg Tyr Asp Ser Arg Cys Pro Ala Arg Leu Leu Asp Val
190                 195                 200                 205

GTC CCA GGT CGT AGT GCT GAT GCT TTA CGG TCC TGG CTT GGC TCC CGC        792
Val Pro Gly Arg Ser Ala Asp Ala Leu Arg Ser Trp Leu Gly Ser Arg
                210                 215                 220
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GGT GAA CAG TTC CGC AAT CAG ATA CGG ATC GTG TCC ATG GAT GGA TTC 840

Gly Glu Gln Phe Arg Asn Gln Ile Arg Ile Val Ser Met Asp Gly Phe

225 230 235

CAA GGC TAC GCC ACA GCA AGT AAA GAA CTC ATT CCT TCT GCT CGT CGC 888

Gln Gly Tyr Ala Thr Ala Ser Lys Glu Leu Ile Pro Ser Ala Arg Arg

240 245 250

GTG ATG GAT CCA TTC CAT GTT GTG CGG CTT GCT GGT GAC AAG CTC ACC 936

Val Met Asp Pro Phe His Val Val Arg Leu Ala Gly Asp Lys Leu Thr

255 260 265

GCC TGC CGG CAA CGC CTC CAG CGG GAG AAA TAC CAG CGT CGT GGT TTA 984

Ala Cys Arg Gln Arg Leu Gln Arg Glu Lys Tyr Gln Arg Arg Gly Leu

270 275 280 285

AGC CAG GAT CCG TTG TAT AAA AAC CGG AAG ACC TTG TTG ACC ACG CAC 1032

Ser Gln Asp Pro Leu Tyr Lys Asn Arg Lys Thr Leu Leu Thr Thr His

290 295 300

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AAG TGG TTG AGT CCT CGT CAG CAA GAA AGC TTG GAG CAG TTG TGG GCG      1080
Lys Trp Leu Ser Pro Arg Gln Gln Glu Ser Leu Glu Gln Leu Trp Ala
            305                 310                 315

TAT GAC AAA GAC TAC GGG GTG TTA AAG CTT GCG TGG CTT GCG TAT CAG      1128
Tyr Asp Lys Asp Tyr Gly Val Leu Lys Leu Ala Trp Leu Ala Tyr Gln
        320                 325                 330

GCG ATT ATT GAT TGT TAT CAG ATG GGT AAT AAG CGT GAA GCG AAG AAG      1176
Ala Ile Ile Asp Cys Tyr Gln Met Gly Asn Lys Arg Glu Ala Lys Lys
    335                 340                 345

AAA ATG CGG ACC ATT ATT GAT CAG CTT CGG GTG TTG AAG GGG CCG AAT      1224
Lys Met Arg Thr Ile Ile Asp Gln Leu Arg Val Leu Lys Gly Pro Asn
350                 355                 360                 365

AAG GAA CTC GCG CAG TTG GGT CGT AGT TTG TTT AAA CGA CTT GGT GAT      1272
Lys Glu Leu Ala Gln Leu Gly Arg Ser Leu Phe Lys Arg Leu Gly Asp
                370                 375                 380
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GTG TTG GCG TAT TTC GAT GTT GGT GTC TCC AAC GGT CCG GTC GAA GCG 1320

Val Leu Ala Tyr Phe Asp Val Gly Val Ser Asn Gly Pro Val Glu Ala

385 390 395

ATC AAC GGA CGG TTG GAG CAT TTG CGT GGG ATT GCT CTA GGT TTC CGT 1368

Ile Asn Gly Arg Leu Glu His Leu Arg Gly Ile Ala Leu Gly Phe Arg

400 405 410

AAT TTG AAC CAC TAC ATT CTG CGG TGC CTT ATC CAT TCA GGG CAG TTG 1416

Asn Leu Asn His Tyr Ile Leu Arg Cys Leu Ile His Ser Gly Gln Leu

415 420 425

GTC CAT AAG ATC AAT GCA CTC TAA AACAGGAAGA GCC 1453

Val His Lys Ile Asn Ala Leu *

430 435

(6) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 437 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met	Lys	Ser	Thr	Gly	Asn	Ile	Ile	Ala	Asp	Thr	Ile	Cys	Arg	Thr	Ala
1				5				10						15	

Glu	Leu	Gly	Leu	Thr	Ile	Thr	Gly	Ala	Ser	Asp	Ala	Gly	Asp	Tyr	Thr
			20				25				30				

Leu	Ile	Glu	Ala	Asp	Ala	Leu	Asp	Tyr	Thr	Ser	Thr	Cys	Pro	Glu	Cys
		35				40				45					

Phe	Gln	Pro	Gly	Val	Phe	Arg	His	His	Thr	His	Arg	Met	Leu	Ile	Asp
	50				55				60						

Leu	Pro	Ile	Val	Gly	Phe	Pro	Thr	Lys	Leu	Phe	Ile	Arg	Leu	Pro	Arg
65				70				75						80	

Tyr Arg Cys Thr Asn Pro Thr Cys Lys Gln Lys Tyr Phe Gln Ala Glu
85 90 95

Leu Ser Cys Ala Asp His Gly Lys Lys Val Thr His Arg Val Thr Arg
100 105 110

Trp Ile Leu Gln Arg Leu Ala Ile Asp Arg Met Ser Val His Ala Thr
115 120 125

Ala Lys Ala Leu Gly Leu Gly Trp Asp Leu Thr Cys Gln Leu Ala Leu
130 135 140

Asp Met Cys Arg Glu Leu Val Tyr Asn Asp Pro His His Leu Asp Gly
145 150 155 160

Val Tyr Val Ile Gly Val Asp Glu His Lys Trp Ser His Asn Arg Ala
165 170 175

Lys His Gly Asp Gly Phe Val Thr Val Ile Val Asp Met Thr Gly His
180 185 190

Arg Tyr Asp Ser Arg Cys Pro Ala Arg Leu Leu Asp Val Val Pro Gly
195 200 205

Arg Ser Ala Asp Ala Leu Arg Ser Trp Leu Gly Ser Arg Gly Glu Gln
210 215 220

Phe Arg Asn Gln Ile Arg Ile Val Ser Met Asp Gly Phe Gln Gly Tyr
225 230 235 240

Ala Thr Ala Ser Lys Glu Leu Ile Pro Ser Ala Arg Arg Val Met Asp
245 250 255

Pro Phe His Val Val Arg Leu Ala Gly Asp Lys Leu Thr Ala Cys Arg
260 265 270

Gln Arg Leu Gln Arg Glu Lys Tyr Gln Arg Arg Gly Leu Ser Gln Asp
275 280 285

Pro Leu Tyr Lys Asn Arg Lys Thr Leu Leu Thr Thr His Lys Trp Leu
290 295 300

Ser Pro Arg Gln Gln Glu Ser Leu Glu Gln Leu Trp Ala Tyr Asp Lys
305 310 315 320

Asp Tyr Gly Val Leu Lys Leu Ala Trp Leu Ala Tyr Gln Ala Ile Ile
325 330 335

Asp Cys Tyr Gln Met Gly Asn Lys Arg Glu Ala Lys Lys Lys Met Arg
340 345 350

Thr Ile Ile Asp Gln Leu Arg Val Leu Lys Gly Pro Asn Lys Glu Leu
355 360 365

Ala Gln Leu Gly Arg Ser Leu Phe Lys Arg Leu Gly Asp Val Leu Ala
370 375 380

Tyr Phe Asp Val Gly Val Ser Asn Gly Pro Val Glu Ala Ile Asn Gly
385 390 395 400

Arg Leu Glu His Leu Arg Gly Ile Ala Leu Gly Phe Arg Asn Leu Asn
405 410 415

His Tyr Ile Leu Arg Cys Leu Ile His Ser Gly Gln Leu Val His Lys
420 425 430

Ile Asn Ala Leu *
435

(7) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Brevibacterium lactofermentum

(B) STRAIN: AJ12036

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGCCCTTCCG GTTTT 15

(8) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Brevibacterium lactofermentum

(B) STRAIN: AJ12036

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGCTCTTCCG GTTTT 15

(9) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1279 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Brevibacterium lactofermentum

(B) STRAIN: AJ12036

(ix) FEATURE:

(A) NAME/KEY: repeat_region

(B) LOCATION: 1..14

(ix) FEATURE:

(A) NAME/KEY: repeat_region

(B) LOCATION: 1266..1279

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGGACTGACC CCTGTTTGGT GGACACCTTG AAACCAGCAT GATGCTGGAA AGGTAATCTG 60

CCACCATGCC ACGCAAGACC TATACAGAGG AGTTCAAGCG CGATGCCGTC GCCTTGTACG 120

AGAACTCCCC AGAGGCTTCG ATCCAGACCA TCGCCACCGA TCTCGGGGTC AACCGCGCCA 180

CGTTGGCGAA CTGGGTGAAA AAATACGGCA CCGCAGGCTC CCAACGAAAC ACCCTCGCCA 240

GCCTCTGTGA ACGAGGCTGA GCAGATCCGG AAACTGGAAC GGGAAAACGC TCGCTTGAGA 300

GAAGAGCGCG ATATCCTGCG GAAAGCTGCA AAATATTTCG CGGAAGAGAC GAATTGGTGA 360

TCCGCTTCCG GTTCGTTGAT GACGCCTCCA AGACCTACTC GGTCAAGCGG ATATGTGACG 420

TCCTCAAACT CAACAGGTCT TCCTACTATA AATGGAAAAG TACCTGCTCA GCACGCAGGA 480

AACGCCTCAT GTCGACGCGA TCCTCGGGGC TCGAGTCAAG GCTGTCTTCA CCACCGAAAA 540

TGGTTGTTAT GGGGCCAAGC GGATCACCGC TGAACTCAAA GACCAGGTGG ATCATGACCC 600

CGTAAATCAC AAGCGGGTCG CTCGGGTGAT GCGCTCGTTG AAGCTGTTTG GCTACACAAA 660

TAAACGCAAG GTCACCACCA CTGTGTCGGA TAAAACCAAG ACAGTGTTTC CTGACCTTGT 720

CGGCCGGAAG TTCACCGCTA ATAAGCCAAA TCAGGTGTAC GTCGGGACAT CACGTACCTG 780

CCGATTGCTG ATGGGTCGAA TATGTACCTG GCTACGGTCA TTGACTGCTA TTCCCGCAGG 840

TTGGTGGGCT TTTCTATCGC ACATCACATG CGTACCTCCC TGGTGCAGAC GCGCTGCTGA 900

TGGCTAAGGG CCAGCGCGAA GCTGACGGGG GCGATCTTTC ACTCGGATCA CGGAAGTGTT 960

TACACTTCTC ACGCATTCCA GACACCTGTA AAGACCTGGG ATAAGGCAGT CGATGGGATC 1020

AATCGGCACC AGTGCGACAA TGCCTCGCGG AGTCCTTCAA CGCAGCACTG AAGCGGAAGT 1080

CCTCCAGGAT TCCAAGACAT TCATGAACCA GTTGCGCTGT CGCCGGGACG TCTTCCGCTG 1140

GTGTACCCGC TACAACATGG TGCGCCGGCA TTCCTGGTGT AAATATCTCG CCCTGCGGTG 1200

TTTGAGAAGC GCTGTCCTGC TATCCTGAAA TCTGCTTCCT GATCAAATCC TCCGTGTCTA 1260

CTATCCGGGG GTCGGGCCC 1279

(10) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Brevibacterium lactofermentum

(B) STRAIN: AJ12036

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGGACTGACC CCTG 14

(11) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Brevibacterium lactofermentum

(B) STRAIN: AJ12036

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGGCCCGACC CCCG 14

(12) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGTTTATT 8

(13) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other..synthetic DNA

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTGGAGCCGA CCATTCCGCG AGG 23

(14) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other..synthetic DNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CCAAAACCGC CCTCCACGGC GAA 23

(15) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 3579 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Brevibacterium lactofermentum

(B) STRAIN: ATCC 13869

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 533..2182

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 2188..3522

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTGGAGCCGA CCATTCCGCG AGGCTGCACT GCAACGAGGT CGTAGTTTTG GTACATGGCT 60

TCTGGCCAGT TCATGGATTG GCTGCCGAAG AAGCTATAGG CATCGCACCA GGGCCACCGA 120

GTTACCGAAG ATGGTGCCGT GCTTTTCGCC TTGGGCAGGG ACCTTGACAA AGCCCACGCT 180

GATATCGCCA AGTGAGGGAT CAGAATAGTG CATGGGCACG TCGATGCTGC CACATTGAGC 240

GGAGGCAATA TCTACCTGAG GTGGGCATTC TTCCCAGCGG ATGTTTTCTT GCGCTGCTGC 300

AGTGGGCATT GATACCAAAA AGGGGCTAAG CGCAGTCGAG GCGGCAAGAA CTGCTACTAC 360

CCTTTTTATT GTCGAACGGG GCATTACGGC TCCAAGGACG TTTGTTTTCT GGGTCAGTTA 420

CCCCAAAAAG CATATACAGA GACCAATGAT TTTTCATTAA AAAGGCAGGG ATTTGTTATA 480

AGTATGGGTC GTATTCTGTG CGACGGGTGT ACCTCGGCTA GAATTTCTCC CC ATG 535

Met

1

ACA CCA GCT GAT CTC GCA ACA TTG ATT AAA GAG ACC GCG GTA GAG GTT 583

Thr Pro Ala Asp Leu Ala Thr Leu Ile Lys Glu Thr Ala Val Glu Val

5 10 15

TTG ACC TCC CGC GAG CTC GAT ACT TCT GTT CTT CCG GAG CAG GTA GTT 631

Leu Thr Ser Arg Glu Leu Asp Thr Ser Val Leu Pro Glu Gln Val Val

20 25 30

GTG GAG CGT CCG CGT AAC CCA GAG CAC GGC GAT TAC GCC ACC AAC ATT 679

Val Glu Arg Pro Arg Asn Pro Glu His Gly Asp Tyr Ala Thr Asn Ile

35 40 45

GCA TTG CAG GTG GCT AAA AAG GTC GGT CAG AAC CCT CGG GAT TTG GCT 727

Ala Leu Gln Val Ala Lys Lys Val Gly Gln Asn Pro Arg Asp Leu Ala

50 55 60 65

ACC TGG CTG GCA GAG GCA TTG GCT GCA GAT GAC GCC ATT GAT TCT GCT 775

Thr Trp Leu Ala Glu Ala Leu Ala Ala Asp Asp Ala Ile Asp Ser Ala

70 75 80

GAA ATT GCT GGC CCA GGC TTT TTG AAC ATT CGC CTT GCT GCA GCA GCA 823

Glu Ile Ala Gly Pro Gly Phe Leu Asn Ile Arg Leu Ala Ala Ala Ala

85 90 95

CAG GGT GAA ATT GTG GCC AAG ATT CTG GCA CAG GGC GAG ACT TTC GGA 871

Gln Gly Glu Ile Val Ala Lys Ile Leu Ala Gln Gly Glu Thr Phe Gly

100 105 110

AAC TCC GAT CAC CTT TCC CAC TTG GAC GTG AAC CTC GAG TTC GTT TCT 919

Asn Ser Asp His Leu Ser His Leu Asp Val Asn Leu Glu Phe Val Ser

115 120 125

GCA AAC CCA ACC GGA CCT ATT CAC CTT GGC GGA ACC CGC TGG GCT GCC 967

Ala Asn Pro Thr Gly Pro Ile His Leu Gly Gly Thr Arg Trp Ala Ala

130 135 140 145

GTG GGT GAC TCT TTG GGT CGT GTG CTG GAG GCT TCC GGC GCG AAA GTG 1015

Val Gly Asp Ser Leu Gly Arg Val Leu Glu Ala Ser Gly Ala Lys Val

150 155 160

ACC CGC GAA TAC TAC TTC AAC GAT CAC GGT CGC CAG ATC GAT CGT TTC 1063

Thr Arg Glu Tyr Tyr Phe Asn Asp His Gly Arg Gln Ile Asp Arg Phe

165 170 175

GCT TTG TCC CTT CTT GCA GCG GCG AAG GGC GAG CCA ACG CCA GAA GAC 1111

Ala Leu Ser Leu Leu Ala Ala Ala Lys Gly Glu Pro Thr Pro Glu Asp

180 185 190

GGT TAT GGC GGC GAA TAC ATT AAG GAA ATT GCG GAG GCA ATC GTC GAA 1159

Gly Tyr Gly Gly Glu Tyr Ile Lys Glu Ile Ala Glu Ala Ile Val Glu

195 200 205

AAG CAT CCT GAA GCG TTG GCT TTG GAG CCT GCC GCA ACC CAG GAG CTT 1207

Lys His Pro Glu Ala Leu Ala Leu Glu Pro Ala Ala Thr Gln Glu Leu

210 215 220 225

TTC CGC GCT GAA GGC GTG GAG ATG ATG TTC GAG CAC ATC AAA TCT TCC 1255

Phe Arg Ala Glu Gly Val Glu Met Met Phe Glu His Ile Lys Ser Ser

230 235 240

CTG CAT GAG TTC GGC ACC GAT TTC GAT GTC TAC TAC CAC GAG AAC TCC 1303

Leu His Glu Phe Gly Thr Asp Phe Asp Val Tyr Tyr His Glu Asn Ser

245 250 255

CTG TTC GAG TCC GGT GCG GTG GAC AAG GCC GTG CAG GTG CTG AAG GAC 1351

Leu Phe Glu Ser Gly Ala Val Asp Lys Ala Val Gln Val Leu Lys Asp

260 265 270

AAC GGC AAC CTG TAC GAA AAC GAG GGC GCT TGG TGG CTG CGT TCC ACC 1399

Asn Gly Asn Leu Tyr Glu Asn Glu Gly Ala Trp Trp Leu Arg Ser Thr

275 280 285

GAA TTC GGC GAT GAC AAA GAC CGC GTG GTG ATC AAG TCT GAC GGC GAC 1447

Glu Phe Gly Asp Asp Lys Asp Arg Val Val Ile Lys Ser Asp Gly Asp

290 295 300 305

GCA GCC TAC ATC GCT GGC GAT ATC GCG TAC GTG GCT GAT AAG TTC TCC 1495

Ala Ala Tyr Ile Ala Gly Asp Ile Ala Tyr Val Ala Asp Lys Phe Ser

310 315 320

CGC GGA CAC AAC CTA AAC ATC TAC ATG TTG GGT GCT GAC CAC CAT GGT 1543

Arg Gly His Asn Leu Asn Ile Tyr Met Leu Gly Ala Asp His His Gly

325 330 335

TAC ATC GCG CGC CTG AAG GCA GCG GCG GCG GCA CTT GGC TAC AAG CCA 1591

Tyr Ile Ala Arg Leu Lys Ala Ala Ala Ala Ala Leu Gly Tyr Lys Pro

340 345 350

GAA GGC GTT GAA GTC CTG ATT GGC CAG ATG GTG AAC CTG CTT CGC GAC 1639

Glu Gly Val Glu Val Leu Ile Gly Gln Met Val Asn Leu Leu Arg Asp

355 360 365

GGC AAG GCA GTG CGT ATG TCC AAG CGT GCA GGC ACC GTG GTC ACC CTA 1687

Gly Lys Ala Val Arg Met Ser Lys Arg Ala Gly Thr Val Val Thr Leu

370 375 380 385

GAT GAC CTC GTT GAA GCA ATC GGC ATC GAT GCG GCG CGT TAC TCC CTG 1735

Asp Asp Leu Val Glu Ala Ile Gly Ile Asp Ala Ala Arg Tyr Ser Leu

390 395 400

ATC CGT TCC TCC GTG GAT TCT TCC CTG GAT ATC GAT CTC GGC CTG TGG 1783

Ile Arg Ser Ser Val Asp Ser Ser Leu Asp Ile Asp Leu Gly Leu Trp

405 410 415

GAA TCC CAG TCC TCC GAC AAC CCT GTG TAC TAC GTG CAG TAC GGA CAC 1831

Glu Ser Gln Ser Ser Asp Asn Pro Val Tyr Tyr Val Gln Tyr Gly His

420 425 430

GCT CGT CTG TGC TCC ATC GCG CGC AAG GCA GAG ACC TTG GGT GTC ACC 1879

Ala Arg Leu Cys Ser Ile Ala Arg Lys Ala Glu Thr Leu Gly Val Thr

435 440 445

GAG GAA GGC GCA GAC CTA TCT CTA CTG ACC CAC GAC CGC GAA GGC GAT 1927

Glu Glu Gly Ala Asp Leu Ser Leu Leu Thr His Asp Arg Glu Gly Asp

450 455 460 465

CTC ATC CGC ACA CTC GGA GAG TTC CCA GCA GTG GTG AAG GCT GCC GCT 1975

Leu Ile Arg Thr Leu Gly Glu Phe Pro Ala Val Val Lys Ala Ala Ala

470 475 480

GAC CTA CGT GAA CCA CAC CGC ATT GCC CGC TAT GCT GAG GAA TTA GCT 2023

Asp Leu Arg Glu Pro His Arg Ile Ala Arg Tyr Ala Glu Glu Leu Ala

485 490 495

GGA ACT TTC CAC CGC TTC TAC GAT TCC TGC CAC ATC CTT CCA AAG GTT 2071

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Gly Thr Phe His Arg Phe Tyr Asp Ser Cys His Ile Leu Pro Lys Val
        500                 505                 510

GAT GAG GAT ACG GCA CCA ATC CAC ACA GCA CGT CTG GCA CTT GCA GCA     2119
Asp Glu Asp Thr Ala Pro Ile His Thr Ala Arg Leu Ala Leu Ala Ala
    515                 520                 525

GCA ACC CGC CAG ACC CTC GCT AAC GCC CTG CAC CTG GTT GGC GTT TCC     2167
Ala Thr Arg Gln Thr Leu Ala Asn Ala Leu His Leu Val Gly Val Ser
530                 535                 540                 545

GCA CCG GAG AAG ATG TAACA ATG GCT ACA GTT GAA AAT TTC AAT GAA       2214
Ala Pro Glu Lys Met       Met Ala Thr Val Glu Asn Phe Asn Glu
                550         1                   5

CTT CCC GCA CAC GTA TGG CCA CGC AAT GCC GTG CGC CAA GAA GAC GGC     2262
Leu Pro Ala His Val Trp Pro Arg Asn Ala Val Arg Gln Glu Asp Gly
 10                  15                  20                  25

GTT GTC ACC GTC GCT GGT GTG CCT CTG CCT GAC CTC GCT GAA GAA TAC     2310
Val Val Thr Val Ala Gly Val Pro Leu Pro Asp Leu Ala Glu Glu Tyr
                30                  35                  40

GGA ACC CCA CTG TTC GTA GTC GAC GAG GAC GAT TTC CGT TCC CGC TGT     2358
Gly Thr Pro Leu Phe Val Val Asp Glu Asp Asp Phe Arg Ser Arg Cys
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45 50 55

CGC GAC ATG GCT ACC GCA TTC GGT GGA CCA GGC AAT GTG CAC TAC GCA 2406
Arg Asp Met Ala Thr Ala Phe Gly Gly Pro Gly Asn Val His Tyr Ala
60 65 70

TCT AAA GCG TTC CTG ACC AAG ACC ATT GCA CGT TGG GTT GAT GAA GAG 2454
Ser Lys Ala Phe Leu Thr Lys Thr Ile Ala Arg Trp Val Asp Glu Glu
75 80 85

GGG CTG GCA CTG GAC ATT GCA TCC ATC AAC GAA CTG GGC ATT GCC CTG 2502
Gly Leu Ala Leu Asp Ile Ala Ser Ile Asn Glu Leu Gly Ile Ala Leu
90 95 100 105

GCC GCT GGT TTC CCC GCC AGC CGT ATC ACC GCG CAC GGC AAC AAC AAA 2550
Ala Ala Gly Phe Pro Ala Ser Arg Ile Thr Ala His Gly Asn Asn Lys
110 115 120

GGC GTA GAG TTC CTG CGC GCG TTG GTT CAA AAC GGT GTG GGA CAC GTG 2598
Gly Val Glu Phe Leu Arg Ala Leu Val Gln Asn Gly Val Gly His Val
125 130 135

GTG CTG GAC TCC GCA CAG GAA CTA GAA CTG TTG GAT TAC GTT GCC GCT 2646
Val Leu Asp Ser Ala Gln Glu Leu Glu Leu Leu Asp Tyr Val Ala Ala
140 145 150

GGT GAA GGC AAG ATT CAG GAC GTG TTG ATC CGC GTA AAG CCA GGC ATC 2694

Gly Glu Gly Lys Ile Gln Asp Val Leu Ile Arg Val Lys Pro Gly Ile

155 160 165

GAA GCA CAC ACC CAC GAG TTC ATC GCC ACT AGC CAC GAA GAC CAG AAG 2742

Glu Ala His Thr His Glu Phe Ile Ala Thr Ser His Glu Asp Gln Lys

170 175 180 185

TTC GGA TTC TCC CTG GCA TCC GGT TCC GCA TTC GAA GCA GCA AAA GCC 2790

Phe Gly Phe Ser Leu Ala Ser Gly Ser Ala Phe Glu Ala Ala Lys Ala

190 195 200

GCC AAC AAC GCA GAA AAC CTG AAC CTG GTT GGC CTG CAC TGC CAC GTT 2838

Ala Asn Asn Ala Glu Asn Leu Asn Leu Val Gly Leu His Cys His Val

205 210 215

GGT TCC CAG GTG TTC GAC GCC GAA GGC TTC AAG CTG GCA GCA GAA CGC 2886

Gly Ser Gln Val Phe Asp Ala Glu Gly Phe Lys Leu Ala Ala Glu Arg

220 225 230

GTG TTG GGC CTG TAC TCA CAG ATC CAC AGC GAA CTG GGC GTT GCC CTT 2934

Val Leu Gly Leu Tyr Ser Gln Ile His Ser Glu Leu Gly Val Ala Leu

235 240 245

CCT GAA CTG GAT CTC GGT GGC GGA TAC GGC ATT GCC TAT ACC GCA GCT 2982

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Pro Glu Leu Asp Leu Gly Gly Gly Tyr Gly Ile Ala Tyr Thr Ala Ala
250                 255                 260                 265

GAA GAA CCA CTC AAC GTC GCA GAA GTT GCC TCC GAC CTG CTC ACC GCA    3030
Glu Glu Pro Leu Asn Val Ala Glu Val Ala Ser Asp Leu Leu Thr Ala
                270                 275                 280

GTC GGA AAA ATG GCA GCG GAA CTA GGC ATC GAC GCA CCA ACC GTG CTT    3078
Val Gly Lys Met Ala Ala Glu Leu Gly Ile Asp Ala Pro Thr Val Leu
            285                 290                 295

GTT GAG CCC GGC CGC GCT ATC GCA GGC CCC TCC ACC GTG ACC ATC TAC    3126
Val Glu Pro Gly Arg Ala Ile Ala Gly Pro Ser Thr Val Thr Ile Tyr
        300                 305                 310

GAA GTC GGC ACC ACC AAA GAC GTC CAC GTA GAC GAC GAC AAA ACC CGC    3174
Glu Val Gly Thr Thr Lys Asp Val His Val Asp Asp Asp Lys Thr Arg
    315                 320                 325

CGT TAC ATC GCC GTG GAC GGA GGC ATG TCC GAC AAC ATC CGC CCA GCA    3222
Arg Tyr Ile Ala Val Asp Gly Gly Met Ser Asp Asn Ile Arg Pro Ala
330                 335                 340                 345

CTC TAC GGC TCC GAA TAC GAC GCC CGC GTA GTA TCC CGC TTC GCC GAA    3270
Leu Tyr Gly Ser Glu Tyr Asp Ala Arg Val Val Ser Arg Phe Ala Glu
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                    350                 355                 360

GGA GAC CCA GTA AGC ACC CGC ATC GTG GGC TCC CAC TGC GAA TCC GGC      3318
Gly Asp Pro Val Ser Thr Arg Ile Val Gly Ser His Cys Glu Ser Gly

                365                 370                 375

GAT ATC CTG ATC AAC GAT GAA ATC TAC CCA TCT GAC ATC ACC AGC GGC      3366
Asp Ile Leu Ile Asn Asp Glu Ile Tyr Pro Ser Asp Ile Thr Ser Gly

        380                 385                 390

GAC TTC CTT GCA CTC GCA GCC ACC GGC GCA TAC TGC TAC GCC ATG AGC      3414
Asp Phe Leu Ala Leu Ala Ala Thr Gly Ala Tyr Cys Tyr Ala Met Ser

    395                 400                 405

TCC CGC TAC AAC GCC TTC ACA CGG CCC GCC GTC GTG TCC GTC CGC GCT      3462
Ser Arg Tyr Asn Ala Phe Thr Arg Pro Ala Val Val Ser Val Arg Ala

410                 415                 420                 425

GGC AGC TCC CGC CTC ATG CTG CGC CGC GAA ACG CTC GAC GAC ATC CTC      3510
Gly Ser Ser Arg Leu Met Leu Arg Arg Glu Thr Leu Asp Asp Ile Leu

                430                 435                 440

TCA CTA GAG GCA TAACGCTTTT CGACGCCTGA CCCCGCCCTT CACCTTCGCC          3562
Ser Leu Glu Ala

            445
```

GTGGAGGGCG GTTTTGG 3579

(16) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other..synthetic DNA

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GTCGACGGAT CGCAAATGGC AAC 23

(17) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other..synthetic DNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGATCCTTGA GCACCTTGCG CAG 23

(18) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1411 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Brevibacterium lactofermentum

(B) STRAIN: ATCC 13869

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 311..1213

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CTCTCGATAT CGAGAGAGAA GCAGCGCCAC GGTTTTTCGG TGATTTTGAG ATTGAAACTT 60

TGGCAGACGG ATCGCAAATG GCAACAAGCC CGTATGTCAT GGACTTTTAA CGCAAAGCTC 120

ACACCCACGA GCTAAAAATT CATATAGTTA AGACAACATT TTTGGCTGTA AAAGACAGCC 180

GTAAAAACCT CTTGCTCATG TCAATTGTTC TTATCGGAAT GTGGCTTGGG CGATTGTTAT 240

GCAAAAGTTG TTAGGTTTTT TGCGGGGTTG TTTAACCCCC AAATGAGGGA AGAAGGTAAC 300

CTTGAACTCT ATG AGC ACA GGT TTA ACA GCT AAG ACC GGA GTA GAG CAC 349

Met Ser Thr Gly Leu Thr Ala Lys Thr Gly Val Glu His

1 5 10

TTC GGC ACC GTT GGA GTA GCA ATG GTT ACT CCA TTC ACG GAA TCC GGA 397

Phe Gly Thr Val Gly Val Ala Met Val Thr Pro Phe Thr Glu Ser Gly

15 20 25

GAC ATC GAT ATC GCT GCT GGC CGC GAA GTC GCG GCT TAT TTG GTT GAT 445

Asp Ile Asp Ile Ala Ala Gly Arg Glu Val Ala Ala Tyr Leu Val Asp

30 35 40 45

AAG GGC TTG GAT TCT TTG GTT CTC GCG GGC ACC ACT GGT GAA TCC CCA 493

Lys Gly Leu Asp Ser Leu Val Leu Ala Gly Thr Thr Gly Glu Ser Pro

50 55 60

ACG ACA ACC GCC GCT GAA AAA CTA GAA CTG CTC AAG GCC GTT CGT GAG 541

Thr Thr Thr Ala Ala Glu Lys Leu Glu Leu Leu Lys Ala Val Arg Glu

65 70 75

GAA GTT GGG GAT CGG GCG AAC GTC ATC GCC GGT GTC GGA ACC AAC AAC 589

Glu Val Gly Asp Arg Ala Asn Val Ile Ala Gly Val Gly Thr Asn Asn

80 85 90

ACG CGG ACA TCT GTG GAA CTT GCG GAA GCT GCT GCT TCT GCT GGC GCA 637

Thr Arg Thr Ser Val Glu Leu Ala Glu Ala Ala Ala Ser Ala Gly Ala

95 100 105

GAC GGC CTT TTA GTT GTA ACT CCT TAT TAC TCC AAG CCG AGC CAA GAG 685

Asp Gly Leu Leu Val Val Thr Pro Tyr Tyr Ser Lys Pro Ser Gln Glu

110 115 120 125

GGA TTG CTG GCG CAC TTC GGT GCA ATT GCT GCA GCA ACA GAG GTT CCA 733
Gly Leu Leu Ala His Phe Gly Ala Ile Ala Ala Ala Thr Glu Val Pro

130 135 140

ATT TGT CTC TAT GAC ATT CCT GGT CGG TCA GGT ATT CCA ATT GAG TCT 781
Ile Cys Leu Tyr Asp Ile Pro Gly Arg Ser Gly Ile Pro Ile Glu Ser

145 150 155

GAT ACC ATG AGA CGC CTG AGT GAA TTA CCT ACG ATT TTG GCG GTC AAG 829
Asp Thr Met Arg Arg Leu Ser Glu Leu Pro Thr Ile Leu Ala Val Lys

160 165 170

GAC GCC AAG GGT GAC CTC GTT GCA GCC ACG TCA TTG ATC AAA GAA ACG 877
Asp Ala Lys Gly Asp Leu Val Ala Ala Thr Ser Leu Ile Lys Glu Thr

175 180 185

GGA CTT GCC TGG TAT TCA GGC GAT GAC CCA CTA AAC CTT GTT TGG CTT 925
Gly Leu Ala Trp Tyr Ser Gly Asp Asp Pro Leu Asn Leu Val Trp Leu

190 195 200 205

GCT TTG GGC GGA TCA GGT TTC ATT TCC GTA ATT GGA CAT GCA GCC CCC 973
Ala Leu Gly Gly Ser Gly Phe Ile Ser Val Ile Gly His Ala Ala Pro

210 215 220

ACA GCA TTA CGT GAG TTG TAC ACA AGC TTC GAG GAA GGC GAC CTC GTC 1021
Thr Ala Leu Arg Glu Leu Tyr Thr Ser Phe Glu Glu Gly Asp Leu Val

225 230 235

CGT GCG CGG GAA ATC AAC GCC AAA CTA TCA CCG CTG GTA GCT GCC CAA 1069
Arg Ala Arg Glu Ile Asn Ala Lys Leu Ser Pro Leu Val Ala Ala Gln

240 245 250

GGT CGC TTG GGT GGA GTC AGC TTG GCA AAA GCT GCT CTG CGT CTG CAG 1117
Gly Arg Leu Gly Gly Val Ser Leu Ala Lys Ala Ala Leu Arg Leu Gln

255 260 265

GGC ATC AAC GTA GGA GAT CCT CGA CTT CCA ATT ATG GCT CCA AAT GAG 1165
Gly Ile Asn Val Gly Asp Pro Arg Leu Pro Ile Met Ala Pro Asn Glu

270 275 280 285

CAG GAA CTT GAG GCT CTC CGA GAA GAC ATG AAA AAA GCT GGA GTT CTA 1213
Gln Glu Leu Glu Ala Leu Arg Glu Asp Met Lys Lys Ala Gly Val Leu

290 295 300

TAAATATGAA TGATTCCCGA AATCGCGGCC GGAAGGTTAC CCGCAAGGCG GCCCACCAGA 1273
AGCTGGTCAG GAAAACCATC TGGATACCCC TGTCTTTCAG GCACCAGATG CTTCCTCTAA 1333
CCAGAGCGCT GTAAAAGCTG AGACCGCCGG AAACGACAAT CGGGATGCTG CGCAAGGTGC 1393
TCAAGGATCC CAACATTC 1411

(19) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other..synthetic DNA

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TCGCGAAGTA GCACCTGTCA CTT 23

(20) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other..synthetic DNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ACGGAATTCA ATCTTACGGC C 21

(21) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1643 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Brevibacterium lactofermentum

(B) STRAIN: ATCC 13869

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TCGCGAAGTA GCACCTGTCA CTTTTGTCTC AAATATTAAA TCGAATATCA ATATACGGTC 60

TGTTTATTGG AACGCATCCC AGTGGCTGAG ACGCATCCGC TAAAGCCCCA GGAACCCTGT 120

GCAGAAAGAA AACACTCCTC TGGCTAGGTA GACACAGTTT ATAAAGGTAG AGTTGAGCGG 180

GTAACTGTCA GCACGTAGAT CGAAAGGTGC ACAAAGGTGG CCCTGGTCGT ACAGAAATAT 240

GGCGGTTCCT CGCTTGAGAG TGCGGAACGC ATTAGAAACG TCGCTGAACG GATCGTTGCC 300

ACCAAGAAGG CTGGAAATGA TGTCGTGGTT GTCTGCTCCG CAATGGGAGA CACCACGGAT 360

GAACTTCTAG AACTTGCAGC GGCAGTGAAT CCCGTTCCGC CAGCTCGTGA AATGGATATG 420

CTCCTGACTG CTGGTGAGCG TATTTCTAAC GCTCTCGTCG CCATGGCTAT TGAGTCCCTT 480

GGCGCAGAAG CTCAATCTTT CACTGGCTGT CAGGCTGGTG TGCTCACCAC CGAGCGCCAC 540

GGAAACGCAC GCATTGTTGA CGTCACACCG GGTCGTGTGC GTGAAGCACT CGATGAGGGC 600

AAGATCTGCA TTGTTGCTGG TTTTCAGGGT GTTAATAAAG AAACCCGCGA TGTCACCACG 660

TTGGGTCGTG GTGGTTCTGA CACCACTGCA GTTGCGTTGG CAGCTGCTTT GAACGCTGAT 720

GTGTGTGAGA TTTACTCGGA CGTTGACGGT GTGTATACCG CTGACCCGCG CATCGTTCCT 780

AATGCACAGA AGCTGGAAAA GCTCAGCTTC GAAGAAATGC TGGAACTTGC TGCTGTTGGC 840

TCCAAGATTT TGGTGCTGCG CAGTGTTGAA TACGCTCGTG CATTCAATGT GCCACTTCGC 900

GTACGCTCGT CTTATAGTAA TGATCCCGGC ACTTTGATTG CCGGCTCTAT GGAGGATATT 960

CCTGTGGAAG AAGCAGTCCT TACCGGTGTC GCAACCGACA AGTCCGAAGC CAAAGTAACC 1020

GTTCTGGGTA TTTCCGATAA GCCAGGCGAG GCTGCCAAGG TTTTCCGTGC GTTGGCTGAT 1080

GCAGAAATCA ACATTGACAT GGTTCTGCAG AACGTCTCCT CTGTGGAAGA CGGCACCACC 1140

GACATCACGT TCACCTGCCC TCGCGCTGAC GGACGCCGTG CGATGGAGAT CTTGAAGAAG 1200

CTTCAGGTTC AGGGCAACTG GACCAATGTG CTTTACGACG ACCAGGTCGG CAAAGTCTCC 1260

CTCGTGGGTG CTGGCATGAA GTCTCACCCA GGTGTTACCG CAGAGTTCAT GGAAGCTCTG 1320

CGCGATGTCA ACGTGAACAT CGAATTGATT TCCACCTCTG AGATCCGCAT TTCCGTGCTG 1380

ATCCGTGAAG ATGATCTGGA TGCTGCTGCA CGTGCATTGC ATGAGCAGTT CCAGCTGGGC 1440

GGCGAAGACG AAGCCGTCGT TTATGCAGGC ACCGGACGCT AAAGTTTTAA AGGAGTAGTT 1500

TTACAATGAC CACCATCGCA GTTGTTGGTG CAACCGGCCA GGTCGGCCAG GTTATGCGCA 1560

CCCTTTTGGA AGAGCGCAAT TTCCCAGCTG ACACTGTTCG TTTCTTTGCT TCCCCGCGTT 1620

CCGCAGGCCG TAAGATTGAA TTC 1643

(22) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other..synthetic DNA

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GGATCCCCAA TCGATACCTG GAA 23

(23) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other..synthetic DNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CGGTTCATCG CCAAGTTTTT CTT 23

(24) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2001 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Brevibacterium lactofermentum

(B) STRAIN: ATCC 13869

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 730..1473

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GGATCCCCAA TCGATACCTG GAACGACAAC CTGATCAGGA TATCCAATGC CTTGAATATT 60

GACGTTGAGG AAGGAATCAC CAGCCATCTC AACTGGAAGA CCTGACGCCT GCTGAATTGG 120

ATCAGTGGCC CAATCGACCC ACCAACCAGG TTGGCTATTA CCGGCGATAT CAAAAACAAC 180

TCGCGTGAAC GTTTCGTGCT CGGCAACGCG GATGCCAGCG ATCGACATAT CGGAGTCACC 240

AACTTGAGCC TGCTGCTTCT GATCCATCGA CGGGGAACCC AACGGCGGCA AAGCAGTGGG 300

GGAAGGGGAG TTGGTGGACT CTGAATCAGT GGGCTCTGAA GTGGTAGGCG ACGGGGCAGC 360

ATCTGAAGGC GTGCGAGTTG TGGTGACCGG GTTAGCGGTT TCAGTTTCTG TCACAACTGG 420

AGCAGGACTA GCAGAGGTTG TAGGCGTTGA GCCGCTTCCA TCACAAGCAC TTAAAAGTAA 480

AGAGGCGGAA ACCACAAGCG CCAAGGAACT ACCTGCGGAA CGGGCGGTGA AGGGCAACTT 540

AAGTCTCATA TTTCAAACAT AGTTCCACCT GTGTGATTAA TCTCCAGAAC GGAACAAACT 600

GATGAACAAT CGTTAACAAC ACAGACCAAA ACGGTCAGTT AGGTATGGAT ATCAGCACCT 660

TCTGAATGGG TACGTCTAGA CTGGTGGGCG TTTGAAAAAC TCTTCGCCCC ACGAAAATGA 720

AGGAGCATA ATG GGA ATC AAG GTT GGC GTT CTC GGA GCC AAA GGC CGT 768

Met Gly Ile Lys Val Gly Val Leu Gly Ala Lys Gly Arg

1 5 10

GTT GGT CAA ACT ATT GTG GCA GCA GTC AAT GAG TCC GAC GAT CTG GAG 816
Val Gly Gln Thr Ile Val Ala Ala Val Asn Glu Ser Asp Asp Leu Glu

15 20 25

CTT GTT GCA GAG ATC GGC GTC GAC GAT GAT TTG AGC CTT CTG GTA GAC 864
Leu Val Ala Glu Ile Gly Val Asp Asp Asp Leu Ser Leu Leu Val Asp

30 35 40 45

AAC GGC GCT GAA GTT GTC GTT GAC TTC ACC ACT CCT AAC GCT GTG ATG 912
Asn Gly Ala Glu Val Val Val Asp Phe Thr Thr Pro Asn Ala Val Met

50 55 60

GGC AAC CTG GAG TTC TGC ATC AAC AAC GGC ATT TCT GCG GTT GTT GGA 960
Gly Asn Leu Glu Phe Cys Ile Asn Asn Gly Ile Ser Ala Val Val Gly

65 70 75

ACC ACG GGC TTC GAT GAT GCT CGT TTG GAG CAG GTT CGC GCC TGG CTT 1008

Thr Thr Gly Phe Asp Asp Ala Arg Leu Glu Gln Val Arg Ala Trp Leu

80 85 90

GAA GGA AAA GAC AAT GTC GGT GTT CTG ATC GCA CCT AAC TTT GCT ATC 1056

Glu Gly Lys Asp Asn Val Gly Val Leu Ile Ala Pro Asn Phe Ala Ile

95 100 105

TCT GCG GTG TTG ACC ATG GTC TTT TCC AAG CAG GCT GCC CGC TTC TTC 1104

Ser Ala Val Leu Thr Met Val Phe Ser Lys Gln Ala Ala Arg Phe Phe

110 115 120 125

GAA TCA GCT GAA GTT ATT GAG CTG CAC CAC CCC AAC AAG CTG GAT GCA 1152

Glu Ser Ala Glu Val Ile Glu Leu His His Pro Asn Lys Leu Asp Ala

130 135 140

CCT TCA GGC ACC GCG ATC CAC ACT GCT CAG GGC ATT GCT GCG GCA CGC 1200

Pro Ser Gly Thr Ala Ile His Thr Ala Gln Gly Ile Ala Ala Ala Arg

145 150 155

AAA GAA GCA GGC ATG GAC GCA CAG CCA GAT GCG ACC GAG CAG GCA CTT 1248
Lys Glu Ala Gly Met Asp Ala Gln Pro Asp Ala Thr Glu Gln Ala Leu

160 165 170

GAG GGT TCC CGT GGC GCA AGC GTA GAT GGA ATC CCA GTT CAC GCA GTC 1296
Glu Gly Ser Arg Gly Ala Ser Val Asp Gly Ile Pro Val His Ala Val

175 180 185

CGC ATG TCC GGC ATG GTT GCT CAC GAG CAA GTT ATC TTT GGC ACC CAG 1344
Arg Met Ser Gly Met Val Ala His Glu Gln Val Ile Phe Gly Thr Gln

190 195 200 205

GGT CAG ACC TTG ACC ATC AAG CAG GAC TCC TAT GAT CGC AAC TCA TTT 1392
Gly Gln Thr Leu Thr Ile Lys Gln Asp Ser Tyr Asp Arg Asn Ser Phe

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                210                 215                 220

GCA CCA GGT GTC TTG GTG GGT GTG CGC AAC ATT GCA CAG CAC CCA GGC     1440
Ala Pro Gly Val Leu Val Gly Val Arg Asn Ile Ala Gln His Pro Gly

            225                 230                 235

CTA GTC GTA GGA CTT GAG CAT TAC CTA GGC CTG TAAAGGCTCA TTTCAGCAGC   1493
Leu Val Val Gly Leu Glu His Tyr Leu Gly Leu

        240                 245

GGGTGGAATT TTTTAAAAGG AGCGTTTAAA GGCTGTGGCC GAACAAGTTA AATTGAGCGT   1553
GGAGTTGATA GCGTGCAGTT CTTTTACTCC ACCCGCTGAT GTTGAGTGGT CAACTGATGT   1613
TGAGGGCGCG GAAGCACTCG TCGAGTTTGC GGGTCGTGCC TGCTACGAAA CTTTTGATAA   1673
GCCGAACCCT CGAACTGCTT CCAATGCTGC GTATCTGCGC CACATCATGG AAGTGGGGCA   1733
CACTGCTTTG CTTGAGCATG CCAATGCCAC GATGTATATC CGAGGCATTT CTCGGTCCGC   1793
GACCCATGAA TTGGTCCGAC ACCGCCATTT TTCCTTCTCT CAACTGTCTC AGCGTTTCGT   1853
GCACAGCGGA GAATCGGAAG TAGTGGTGCC CACTCTCATC GATGAAGATC CGCAGTTGCG   1913
TGAACTTTTC ATGCACGCCA TGGATGAGTC TCGGTTCGCT TTCAATGAGC TGCTTAATGC   1973
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GCTGGAAGAA AAACTTGGCG ATGAACCG 2001

(25) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other..synthetic DNA

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CCGGACAGCT CACCCACAAA ATCAATGCAC TCTAAAAAGG TACCT 45

(26) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other..synthetic DNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CTAGAGGTAC CTTTTTAGAG TGCATTGATT TTGTGGGTGA GCTGT 45

(27) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 34 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other..synthetic DNA

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CTAGCTCGAG ATATCAGATC TACTAGTCGA CCGC 34

(28) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D). TOPOLOGY: linear

(ii) MOLECULE TYPE: other..synthetic DNA

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GGTCGACTAG TAGATCTGAT ATCTCGAG 28

Brief Description of the Drawings

Fig. 1 is a view showing structures of various artificial transposons. Kmr represents a neomycin phosphotransferase gene (kanamycin resistance gene), and Tnp represents a transposase gene. The black-colored portion indicates an inverted repeat sequence.

Fig. 2 is a view showing construction of plasmids pHTN7141 and pHTN7142 each containing the artificial transposon.

Fig. 3 is a view showing construction of plasmid pHTN7143 containing the artificial transposon.

Fig. 4 is a view showing construction of plasmid pHTN7144 containing the artificial transposon.

Fig. 5 is a view showing construction of plasmids pHIS714K1 and pHIS714K2.

Fig. 6 is a view showing construction of plasmid pHTN7145 containing the artificial transposon.

Fig. 7 is a view showing construction of plasmid pHTN7151 containing the artificial transposon.

Fig. 8 is a view showing construction of plasmid pHTN7152 containing the artificial transposon.

Fig. 9 is a view showing construction of plasmid pHTN7156-C containing the artificial transposon.

Fig. 10 is a view showing construction of plasmid pORF1.

Fig. 11 is a view showing construction of plasmid pORF3 and pORF4.

Fig. 12 is a view showing construction of plasmid pORF7.

Fig. 13 is a view showing construction of plasmid pORF8.

Fig. 14 is a view showing construction of plasmid pORF41 containing the transposon unit.

Fig. 15 is a view showing construction of plasmid pORFC0.

Fig. 16 shows the difference between an insertion sequence, an artificial transposon and a transposon unit. TCr means a tetracycline resistant gene, Tnp means a transposase gene and the black-box means an inverted repeat sequence (IR). The dot-underlined portion under the structure figures indicates a region to be transposed.

Fig. 17 is a view showing construction of plasmid pORF40.

Fig. 18 is a view showing construction of plasmid pVK7.

Fig. 19 is a view showing construction of plasmid pVC7.

Fig. 20 is a view showing construction of plasmid p399LYSA and plasmid p299LYSA.

Fig. 21 is a view showing construction of plasmid pABLm.

Fig. 22 is a view showing construction of plasmid pCRDAPA.

Fig. 23 is a view showing construction of plasmid p399DPS.

Fig. 24 is a view showing construction of plasmid p399AK9.

Fig. 25 is a view showing construction of plasmid pCRDAPB.

Fig. 26 is a view showing construction of plasmid p399CAB and pCAB.

Fig. 27 is a view showing construction of plasmid pCABL.

Fig. 28 is a view showing construction of plasmid pHTN7150A.

Fig. 29 is a view showing construction of plasmid pCBLmc.

Fig. 30 is a view showing construction of plasmid pHTN7150.

Claims

1. A method of amplifying a desired gene, which comprises forming an artificial transposon which has a structure that a drug resistance gene and the desired gene are held between inverted repeats and which is transposable within a coryneform bacterium cell, transducing said artificial transposon into the coryneform bacterium cell, transposing

said transposon into the chromosome of the coryneform bacterium, and transducing and amplifying said desired gene in said chromosome.

2. The method of claim 1, wherein the artificial transposon has a structure that a transposase is further held between the inverted repeats.

3. The method of claim 1 or 2, wherein the inverted repeat and the transposase gene are derived from an insertion sequence of a coryneform bacterium.

4. The method of claim 3, wherein the insertion sequence has a base sequence represented by any one of Sequence Nos. 1,5 or 9 of Sequence Table.

5. The method of any one of claims 1 to 4, wherein the drug resistance gene is a chloramphenicol resistance gene or a tetracycline resistance gene.

6. The method of any one of claims 1 to 5, wherein the desired gene is a gene that participates in amino-acid biosynthesis.

7. The method of claim 6, wherein the desired gene is an aspartokinase gene and/or a dihydropicolinic acid synthetase gene.

8. A coryneform bacterium which is formed by transducing the desired gene into the chromosome by the method of any one of claims 1 to 7.

9. A method of producing an amino acid, which comprises incubating a coryneform bacterium formed by transducing the gene that participates in amino-acid biosynthesis into the chromosome by the method of claim 6 in a culture medium to form and accumulate the amino acid in the culture medium, and recovering said amino acid.

10. The method of claim 9, wherein the gene that participates in amino-acid biosynthesis is an aspartokinase gene and/or a dihydropicolinic acid synthetase gene, and the amino acid is lysine.

Fig. 1

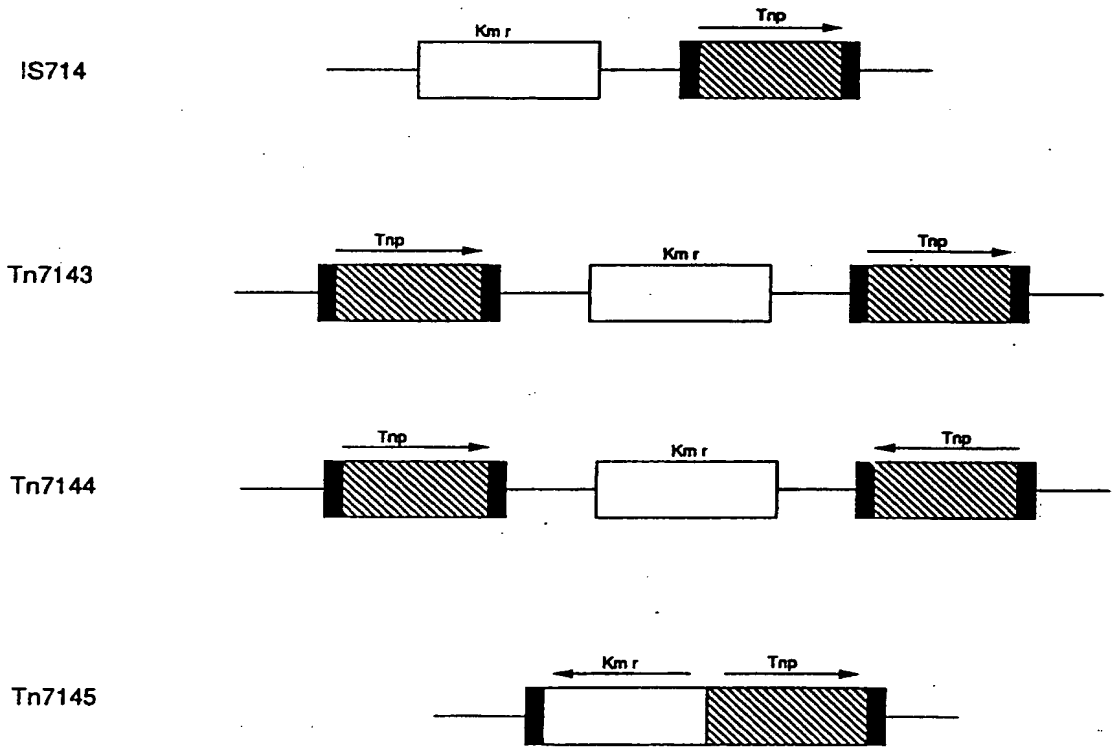

IS714
Km r
Tnp
Tn7143
Tnp
Km r
Tnp
Tn7144
Tnp
Km r
Tnp
Tn7145
Km r
Tnp

Fig. 2

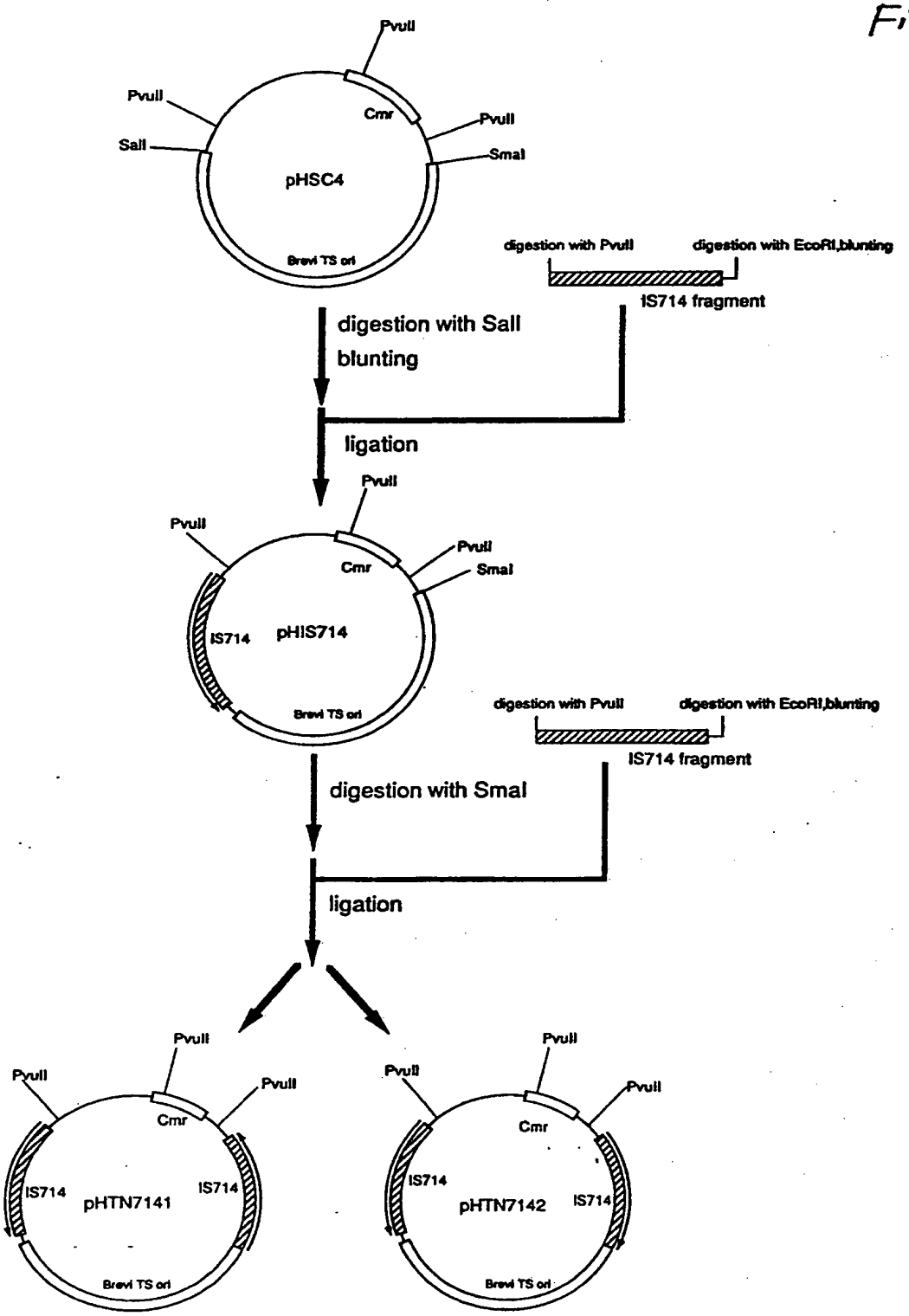

PvuII
PvuII
SalI
Cmr
PvuII
SmaI
pHSC4
Brevi TS ori
digestion with PvuII
digestion with EcoRI,blunting
IS714 fragment
digestion with SalI
blunting
ligation
PvuII
PvuII
Cmr
PvuII
SmaI
IS714
pHIS714
Brevi TS ori
digestion with PvuII
digestion with EcoRI,blunting
IS714 fragment
digestion with SmaI
ligation
PvuII
PvuII
PvuII
Cmr
IS714
IS714
pHTN7141
Brevi TS ori
PvuII
PvuII
PvuII
Cmr
IS714
IS714
pHTN7142
Brevi TS ori

Fig. 3

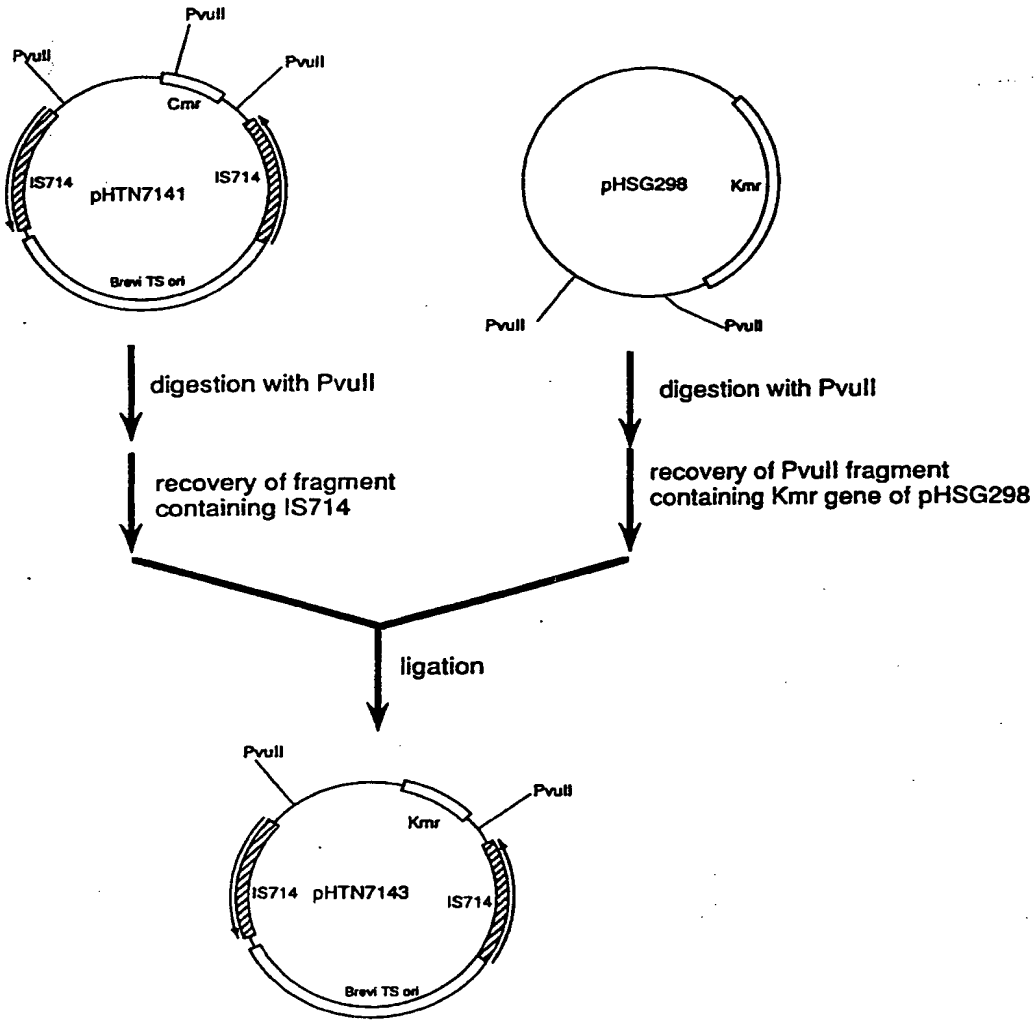
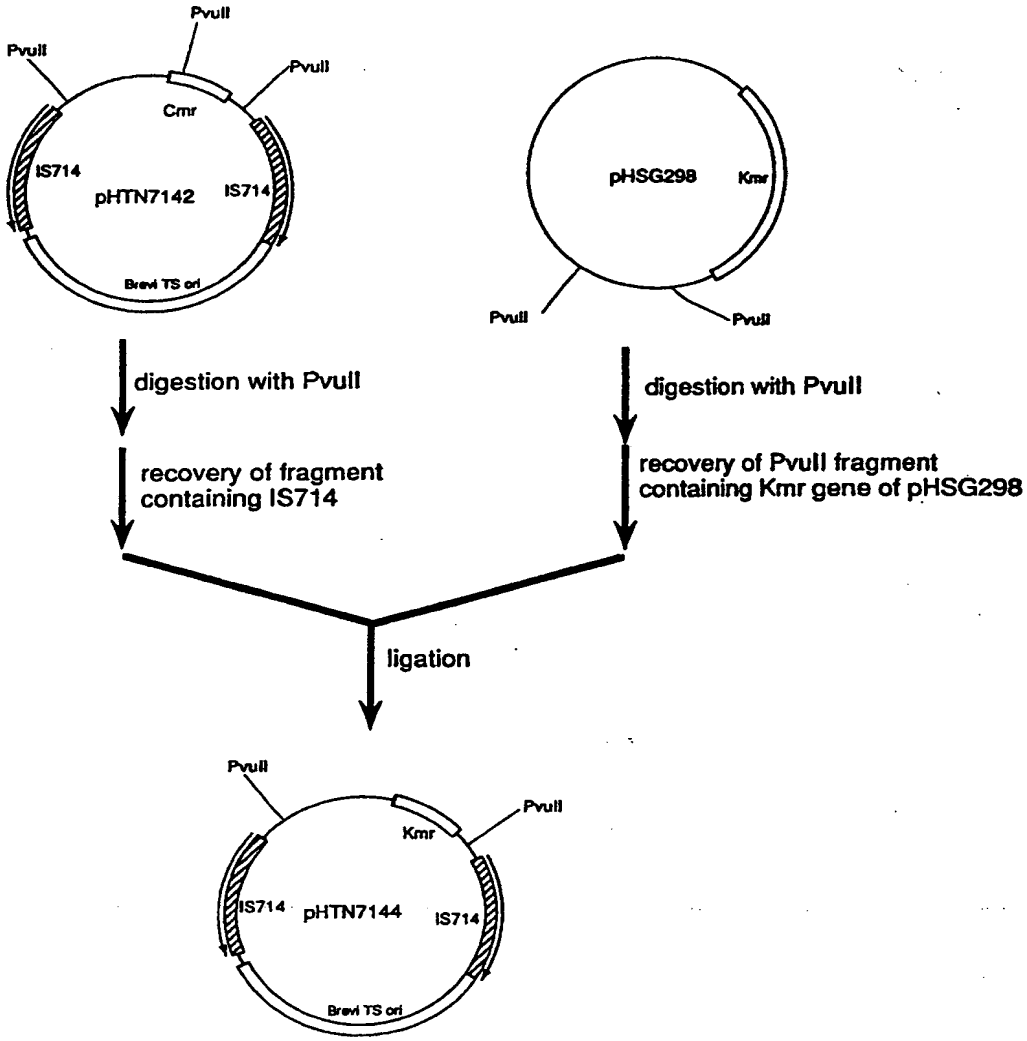

PvuII
PvuII
PvuII
Cmr
IS714
pHTN7141
IS714
Brevi TS ori
pHSG298
Kmr
PvuII
PvuII
digestion with PvuII
recovery of fragment containing IS714
digestion with PvuII
recovery of PvuII fragment containing Kmr gene of pHSG298
ligation
PvuII
PvuII
Kmr
IS714
pHTN7143
IS714
Brevi TS ori

Fig. 4


PvuII
PvuII
PvuII
Cmr
IS714
pHTN7142
IS714
BrevI TS ori
pHSG298
Kmr
PvuII
PvuII
digestion with PvuII
recovery of fragment containing IS714
digestion with PvuII
recovery of PvuII fragment containing Kmr gene of pHSG298
ligation
PvuII
PvuII
Kmr
IS714
pHTN7144
IS714
BrevI TS ori

Fig, 5

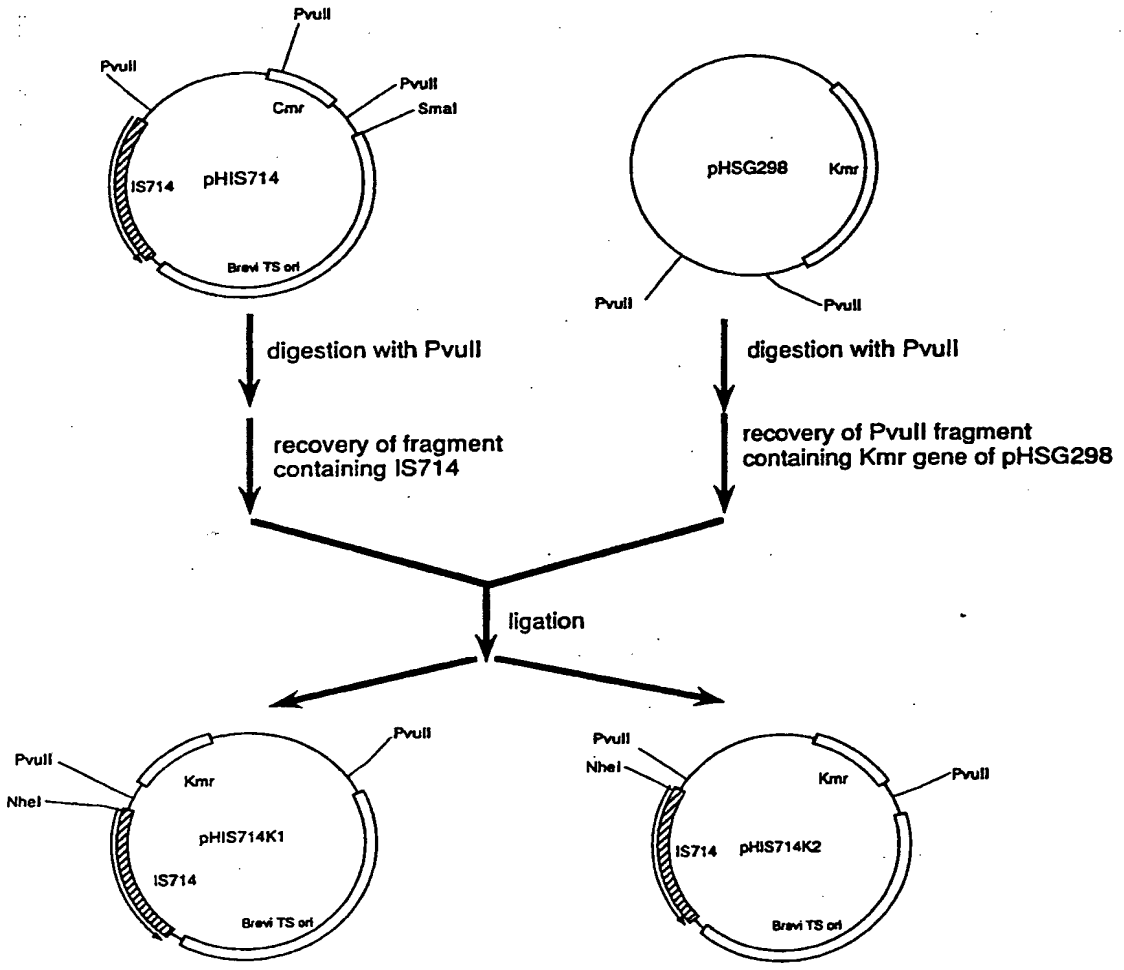

PvuII
PvuII
PvuII
SmaI
Cmr
IS714
pHIS714
Brevi TS ori
pHSG298
Kmr
PvuII
PvuII
digestion with PvuII
digestion with PvuII
recovery of fragment containing IS714
recovery of PvuII fragment containing Kmr gene of pHSG298
ligation
PvuII
NheI
Kmr
PvuII
pHIS714K1
IS714
Brevi TS ori
PvuII
NheI
Kmr
PvuII
IS714
pHIS714K2
Brevi TS ori

Fig. 6

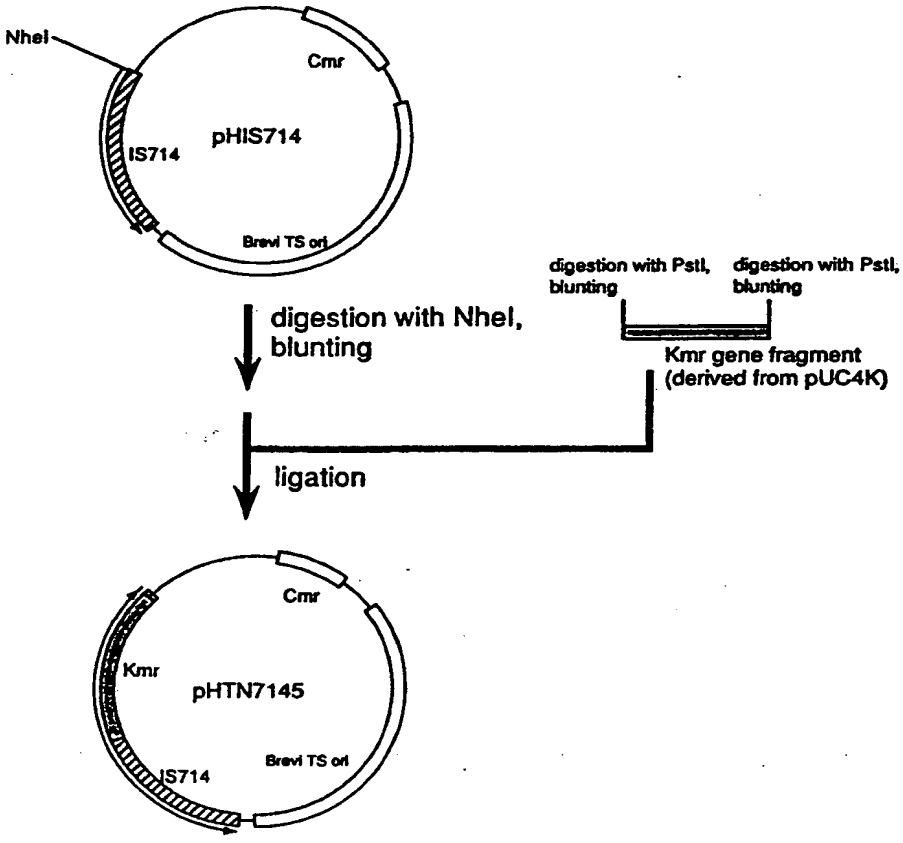

NheI
Cmr
pHIS714
IS714
Brevi TS ori
digestion with NheI, blunting
digestion with PstI, blunting
digestion with PstI, blunting
Kmr gene fragment (derived from pUC4K)
ligation
Cmr
Kmr
pHTN7145
Brevi TS ori
IS714

Fig.7

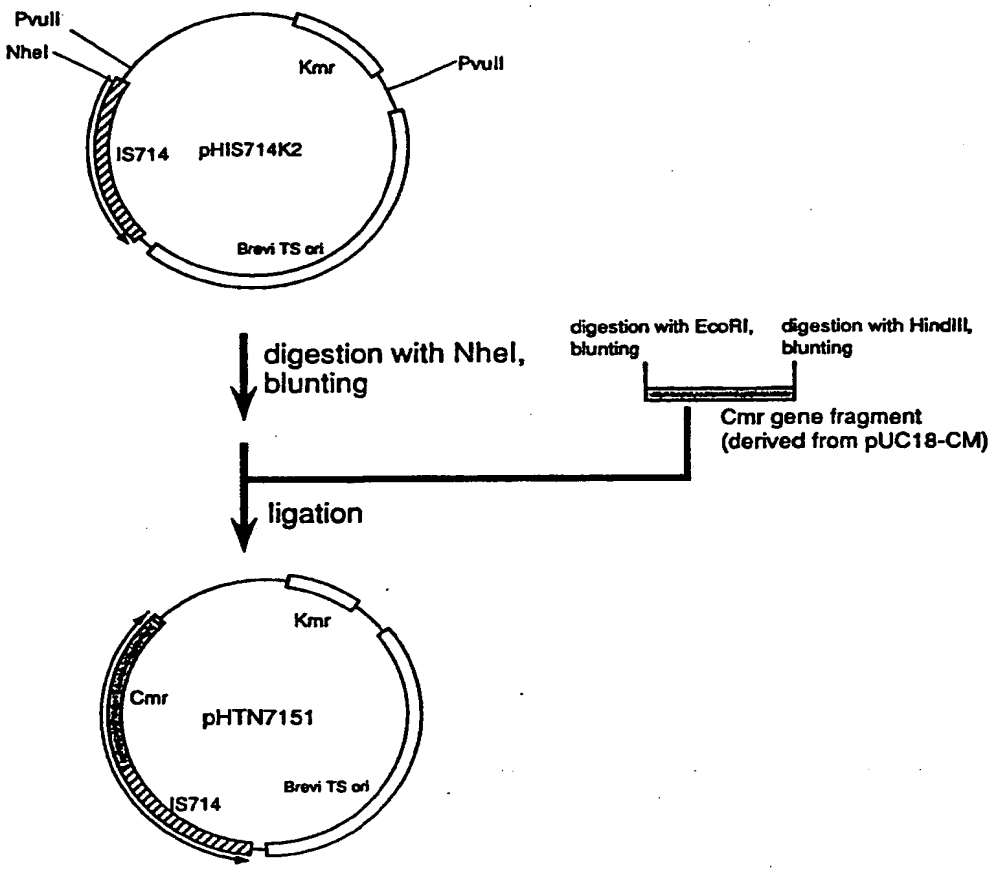

PvuII
NheI
Kmr
PvuII
IS714
pHIS714K2
Brevi TS ori
digestion with NheI, blunting
digestion with EcoRI, blunting
digestion with HindIII, blunting
Cmr gene fragment (derived from pUC18-CM)
ligation
Kmr
Cmr
pHTN7151
Brevi TS ori
IS714

Fig. 8

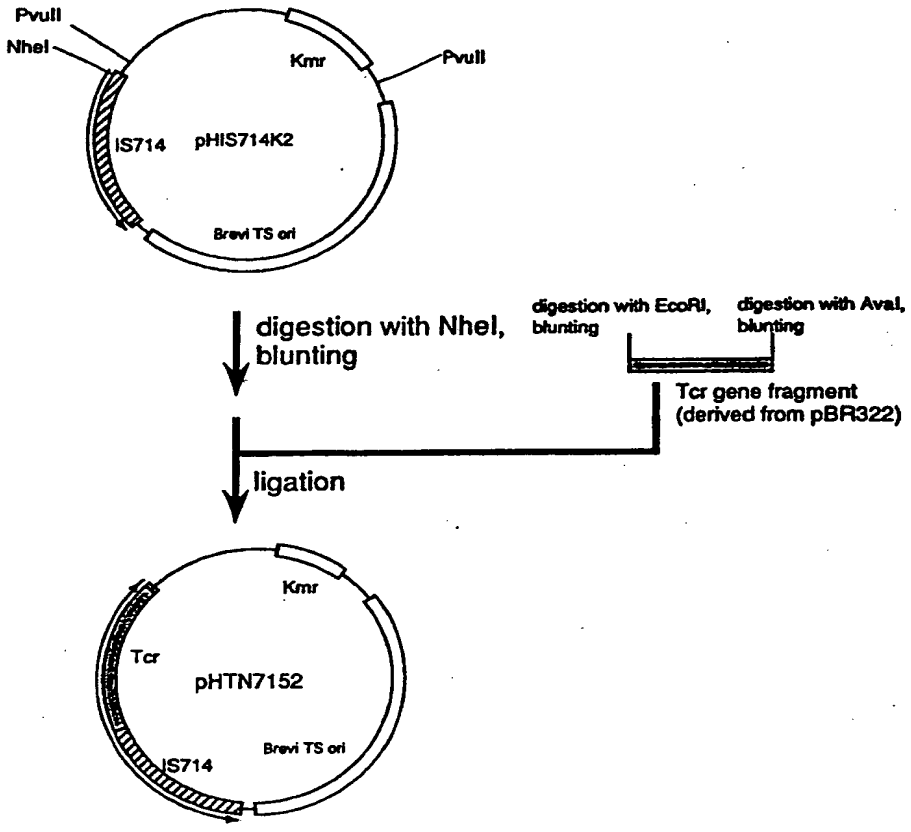

PvuII
NheI
Kmr
PvuII
IS714
pHIS714K2
Brevi TS ori
digestion with NheI, blunting
digestion with EcoRI, blunting
digestion with AvaI, blunting
Tcr gene fragment (derived from pBR322)
ligation
Kmr
Tcr
pHTN7152
Brevi TS ori
IS714

Fig. 9

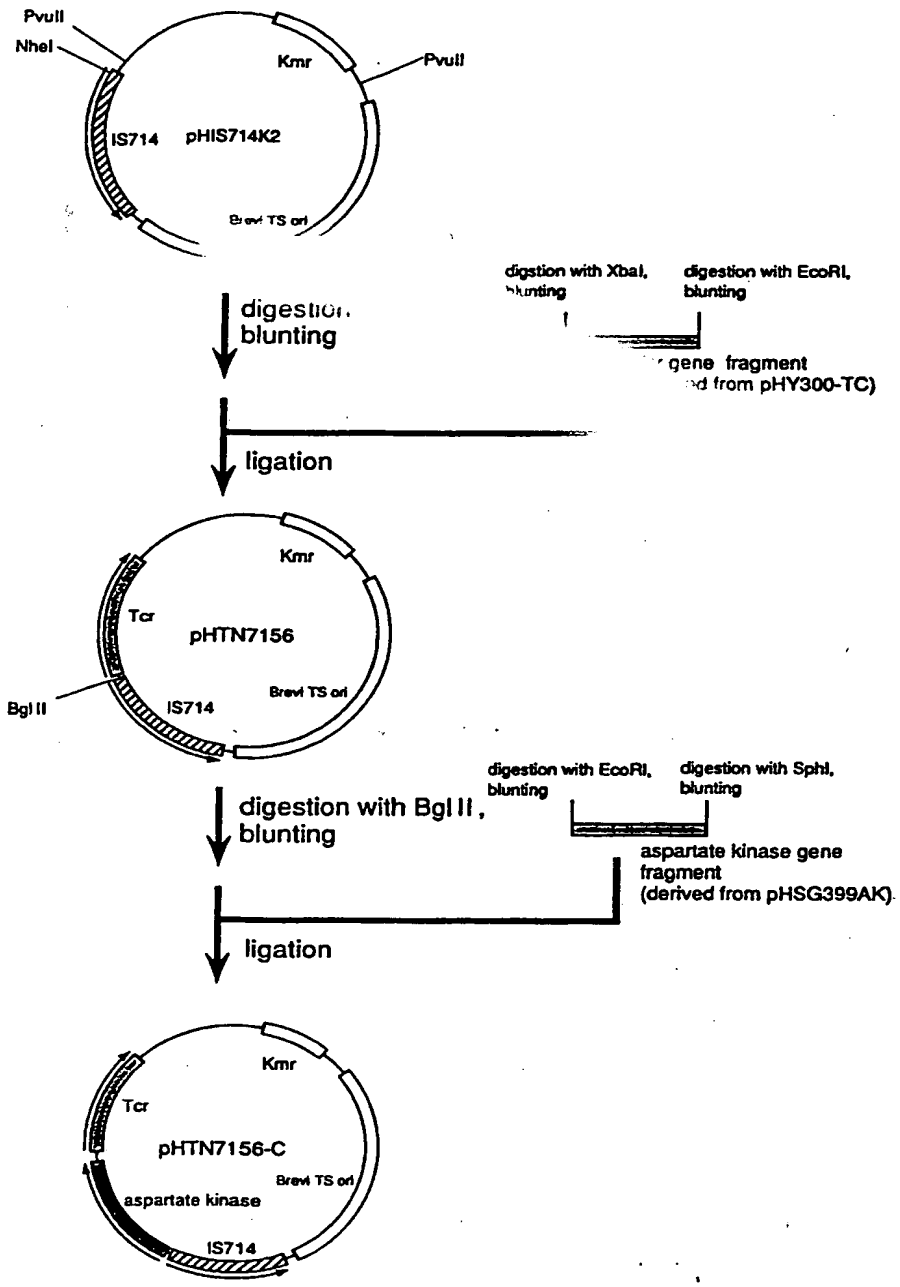
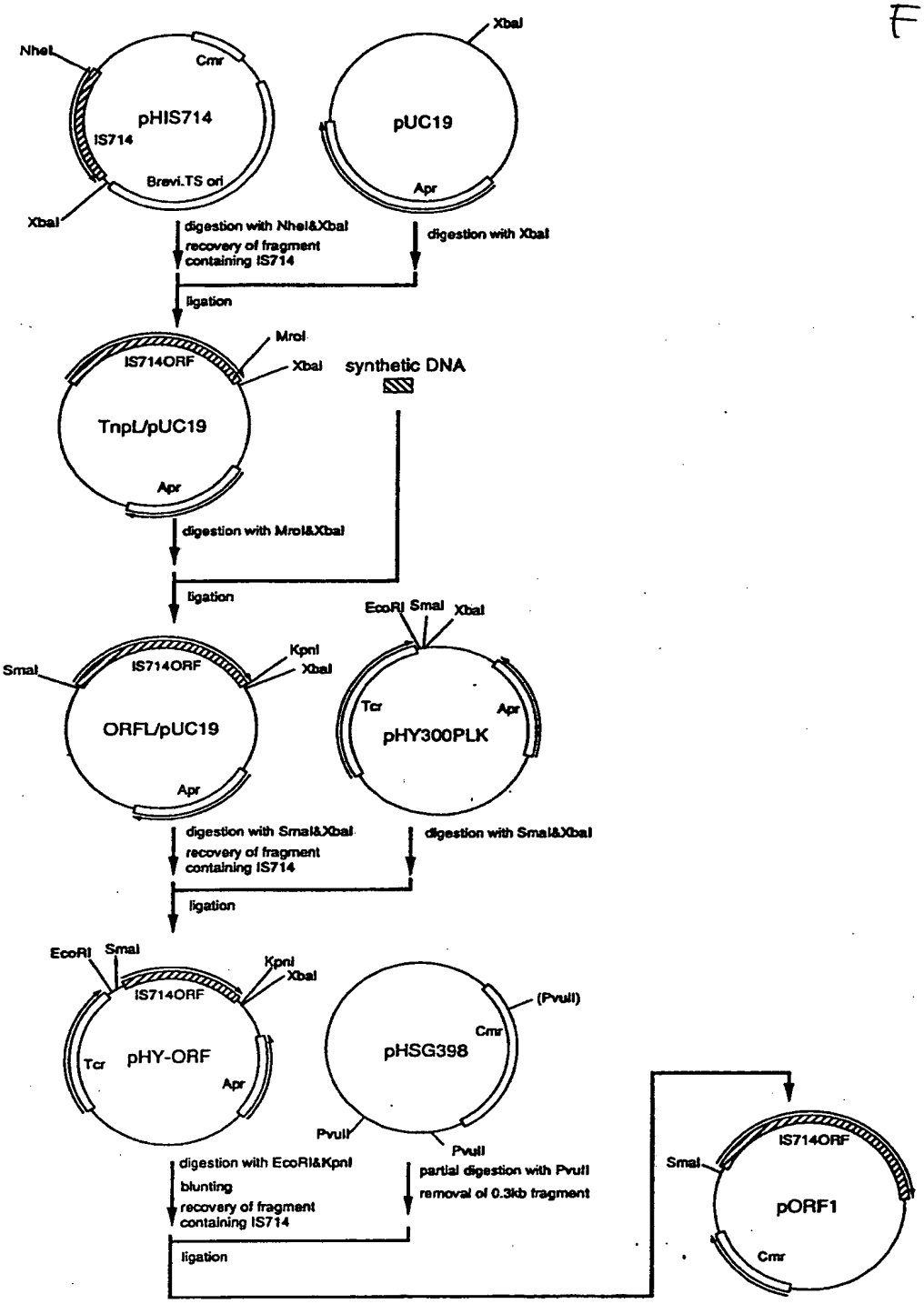

PvuII
NheI
Kmr
PvuII
IS714
pHIS714K2
digestion with XbaI, blunting
digestion with EcoRI, blunting
digestion, blunting
gene fragment
from pHY300-TC)
ligation
Kmr
Tcr
pHTN7156
BglII
IS714
digestion with EcoRI, blunting
digestion with SphI, blunting
digestion with BglII, blunting
aspartate kinase gene fragment
(derived from pHSG399AK)
ligation
Kmr
Tcr
pHTN7156-C
aspartate kinase
IS714

Fig. 10


NheI
Cmr
pHIS714
IS714
Brevi.TS ori
XbaI
XbaI
pUC19
Apr
digestion with NheI&XbaI
recovery of fragment
containing IS714
digestion with XbaI
ligation
MroI
IS714ORF
XbaI
synthetic DNA
TnpL/pUC19
Apr
digestion with MroI&XbaI
ligation
EcoRI SmaI XbaI
SmaI
IS714ORF
KpnI
XbaI
ORFL/pUC19
Apr
Tcr
Apr
pHY300PLK
digestion with SmaI&XbaI
recovery of fragment
containing IS714
digestion with SmaI&XbaI
ligation
EcoRI
SmaI
KpnI
XbaI
IS714ORF
Tcr
pHY-ORF
Apr
(PvuII)
Cmr
pHSG398
PvuII
PvuII
digestion with EcoRI&KpnI
blunting
recovery of fragment
containing IS714
partial digestion with PvuII
removal of 0.3kb fragment
ligation
IS714ORF
SmaI
pORF1
Cmr

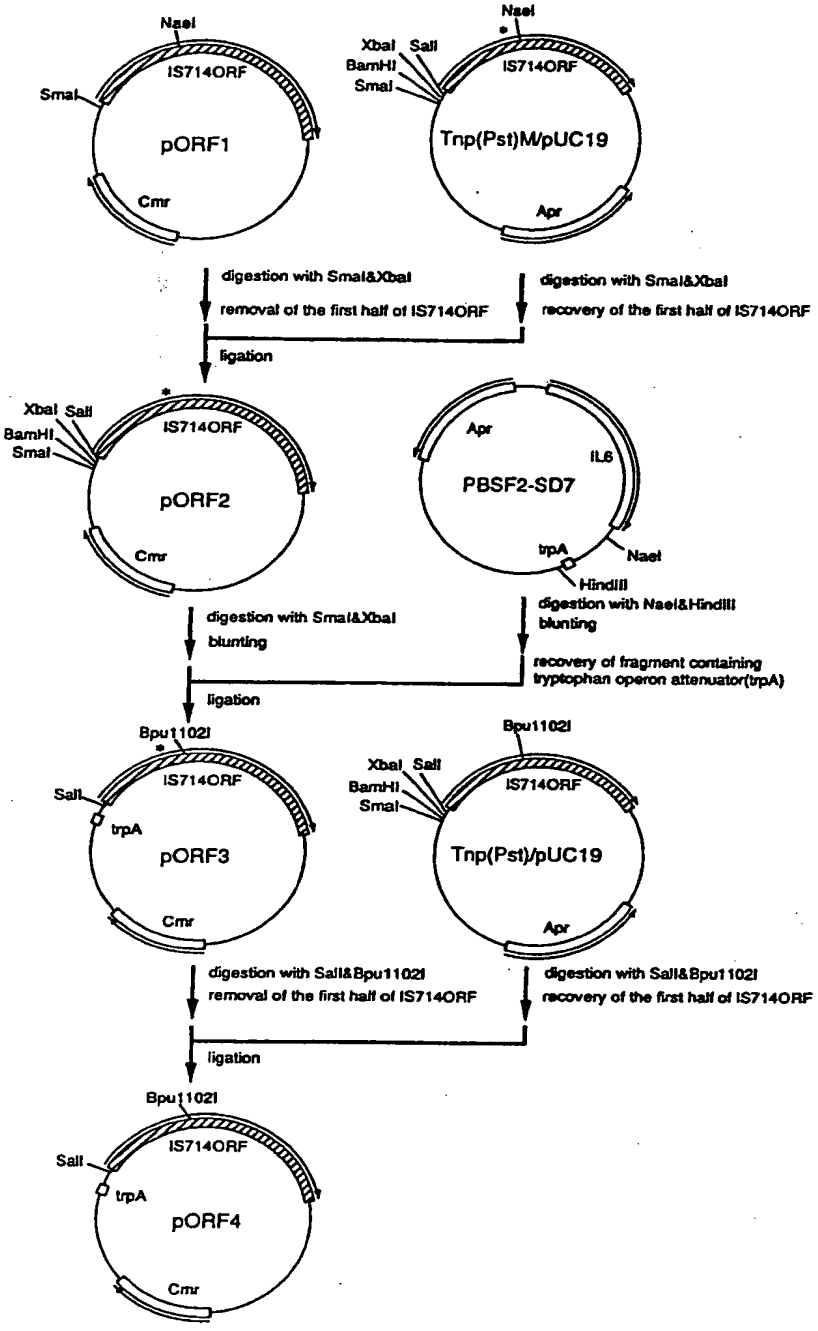

NaeI
IS714ORF
SmaI
pORF1
CmR
NaeI
XbaI SalI
BamHI
SmaI
IS714ORF
Tnp(Pst)M/pUC19
ApR
digestion with SmaI&XbaI
removal of the first half of IS714ORF
digestion with SmaI&XbaI
recovery of the first half of IS714ORF
ligation
XbaI SalI
BamHI
SmaI
IS714ORF
pORF2
CmR
ApR
IL6
PBSF2-SD7
trpA
NaeI
HindIII
digestion with SmaI&XbaI
blunting
digestion with NaeI&HindIII
blunting
recovery of fragment containing
tryptophan operon attenuator(trpA)
ligation
Bpu1102I
SalI
IS714ORF
trpA
pORF3
CmR
Bpu1102I
XbaI SalI
BamHI
SmaI
IS714ORF
Tnp(Pst)/pUC19
ApR
digestion with SalI&Bpu1102I
removal of the first half of IS714ORF
digestion with SalI&Bpu1102I
recovery of the first half of IS714ORF
ligation
Bpu1102I
SalI
IS714ORF
trpA
pORF4
CmR

Fig. 11

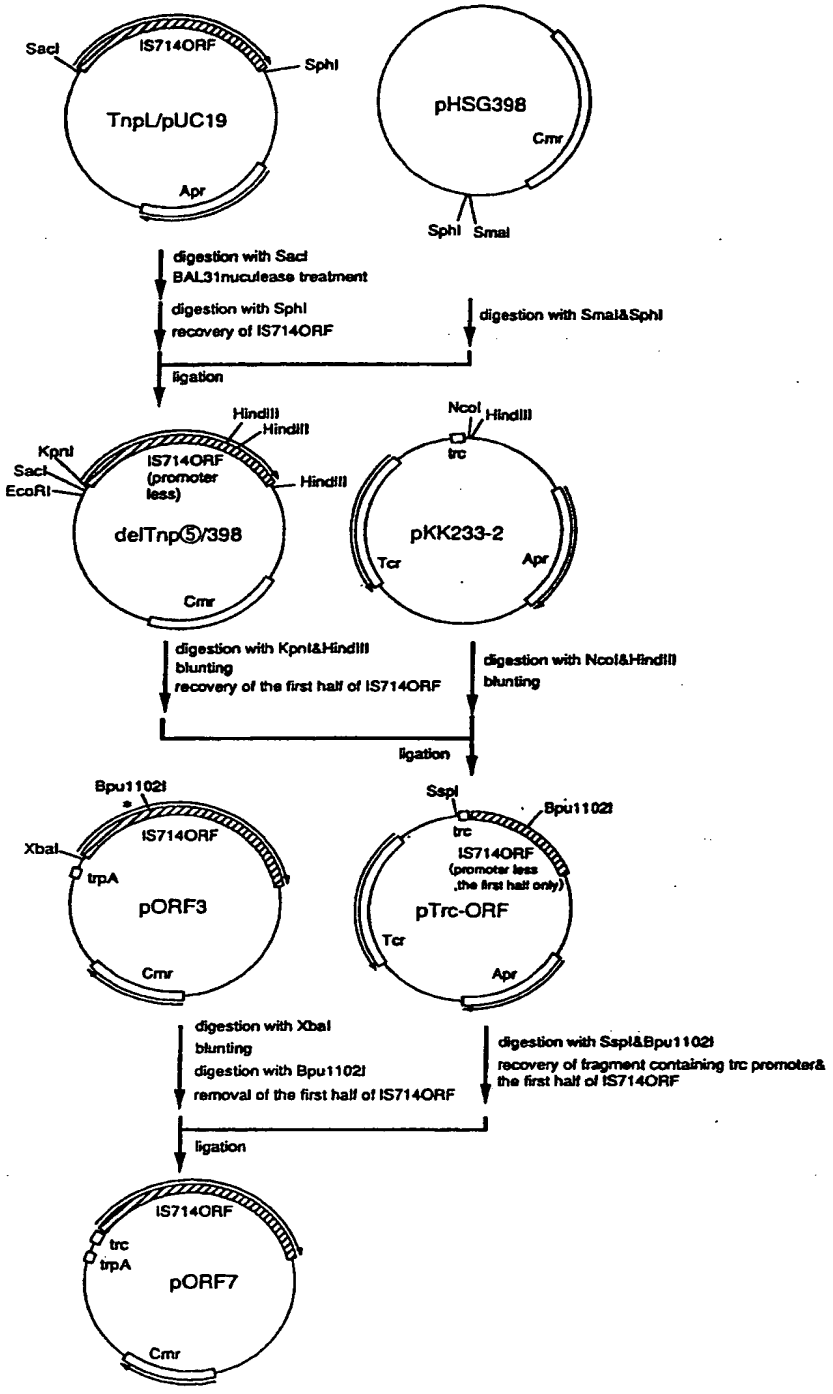

SacI
IS714ORF
SphI
TnpL/pUC19
Apr
pHSG398
Cmr
SphI
SmaI
digestion with SacI
BAL31nuculease treatment
digestion with SphI
recovery of IS714ORF
digestion with SmaI&SphI
ligation
HindIII
HindIII
KpnI
SacI
EcoRI
IS714ORF
(promoter less)
HindIII
delTnp⑤/398
Cmr
NcoI
HindIII
trc
pKK233-2
Tcr
Apr
digestion with KpnI&HindIII
blunting
recovery of the first half of IS714ORF
digestion with NcoI&HindIII
blunting
ligation
Bpu1102I
IS714ORF
XbaI
trpA
pORF3
Cmr
SspI
Bpu1102I
trc
IS714ORF
(promoter less
,the first half only)
pTrc-ORF
Tcr
Apr
digestion with XbaI
blunting
digestion with Bpu1102I
removal of the first half of IS714ORF
digestion with SspI&Bpu1102I
recovery of fragment containing trc promoter&
the first half of IS714ORF
ligation
IS714ORF
trc
trpA
pORF7
Cmr

Fig. 12

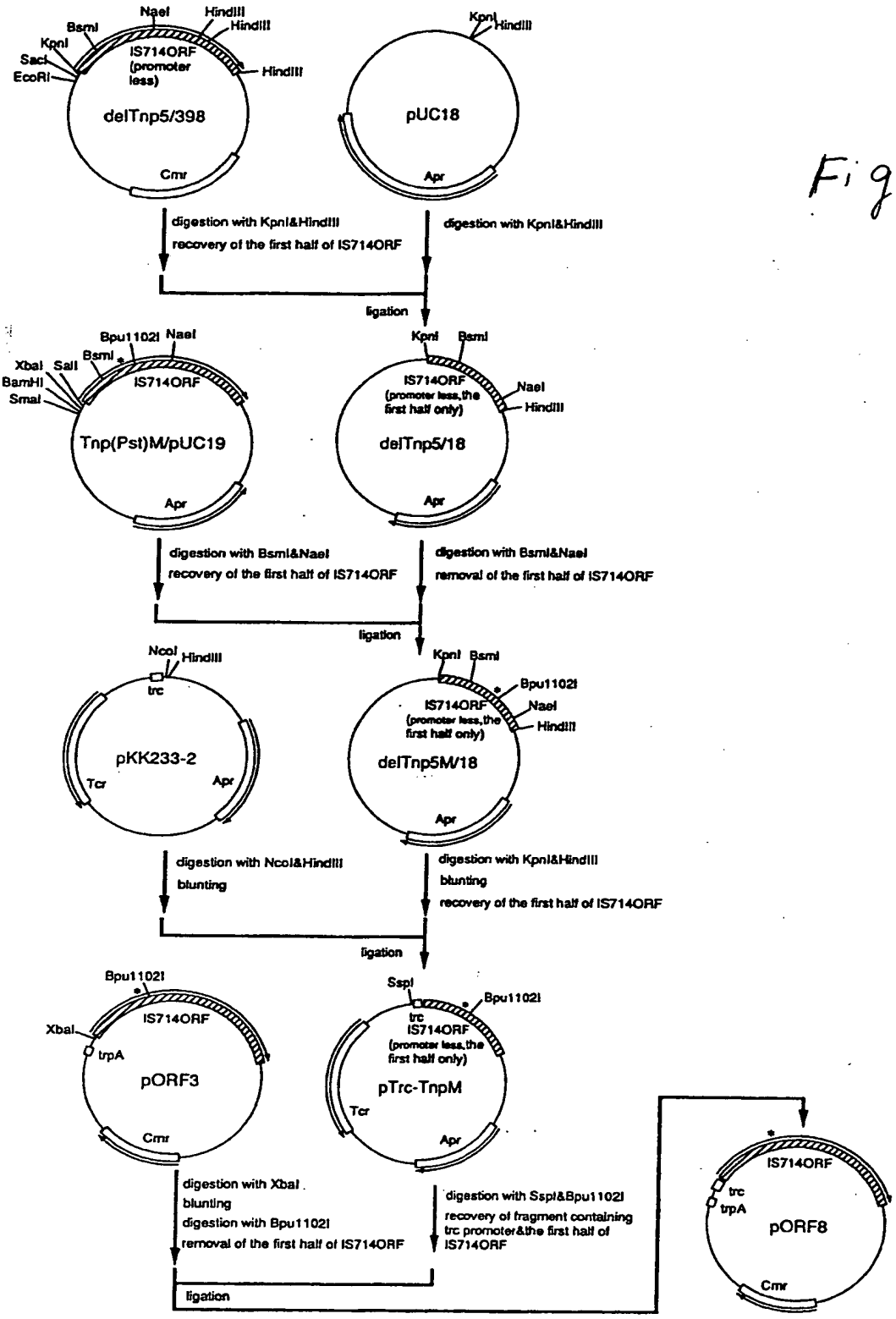

NaeI
HindIII
HindIII
BsmI
KpnI
SacI
EcoRI
IS714ORF
(promoter less)
HindIII
delTnp5/398
Cmr
KpnI
HindIII
pUC18
Apr
digestion with KpnI&HindIII
recovery of the first half of IS714ORF
digestion with KpnI&HindIII
ligation
Bpu1102I
NaeI
BsmI
XbaI
SalI
BamHI
SmaI
IS714ORF
Tnp(Pst)M/pUC19
Apr
KpnI
BsmI
IS714ORF
(promoter less,the first half only)
NaeI
HindIII
delTnp5/18
Apr
digestion with BsmI&NaeI
recovery of the first half of IS714ORF
digestion with BsmI&NaeI
removal of the first half of IS714ORF
ligation
NcoI
HindIII
trc
pKK233-2
Tcr
Apr
KpnI
BsmI
Bpu1102I
IS714ORF
(promoter less,the first half only)
NaeI
HindIII
delTnp5M/18
Apr
digestion with NcoI&HindIII
blunting
digestion with KpnI&HindIII
blunting
recovery of the first half of IS714ORF
ligation
Bpu1102I
IS714ORF
XbaI
trpA
pORF3
Cmr
SspI
Bpu1102I
trc
IS714ORF
(promoter less,the first half only)
pTrc-TnpM
Tcr
Apr
digestion with XbaI
blunting
digestion with Bpu1102I
removal of the first half of IS714ORF
digestion with SspI&Bpu1102I
recovery of fragment containing trc promoter&the first half of IS714ORF
ligation
IS714ORF
trc
trpA
pORF8
Cmr

Fig. 13

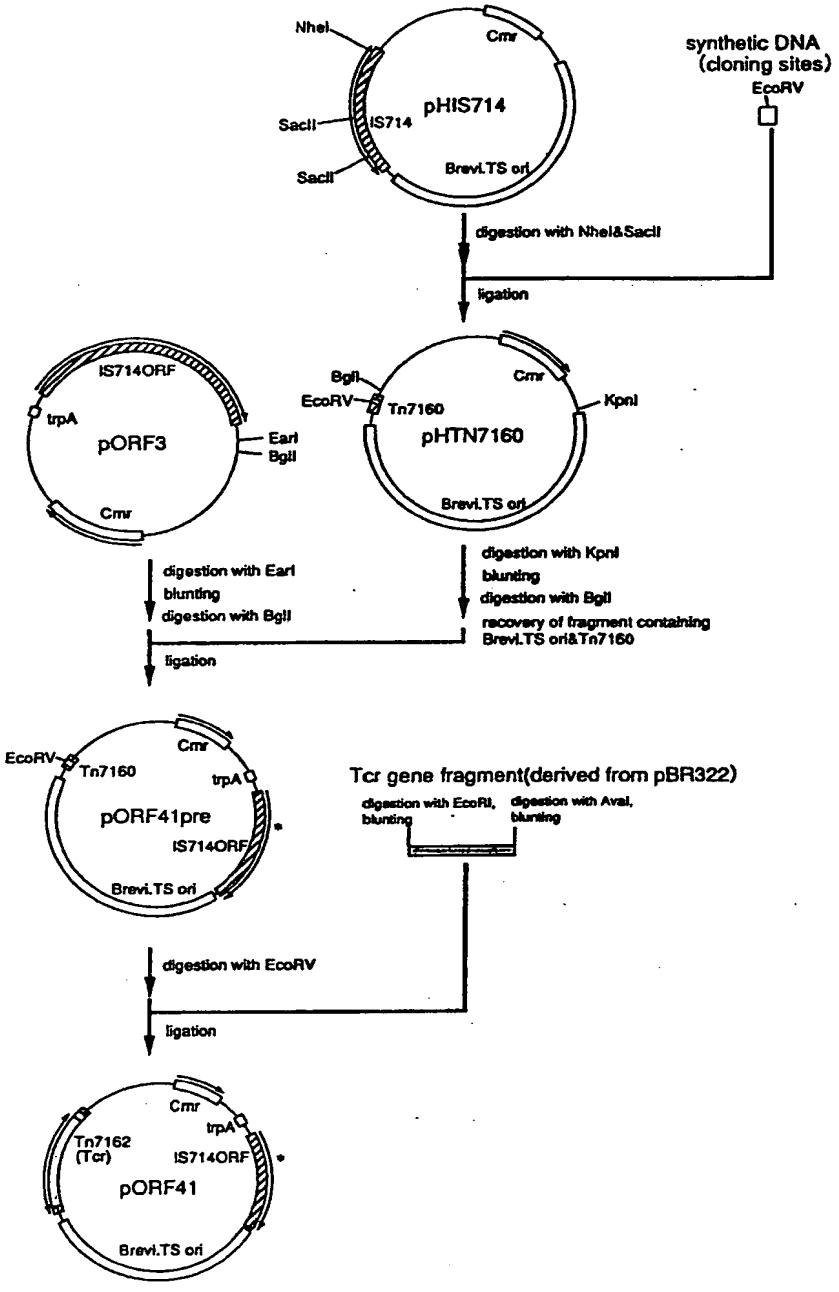
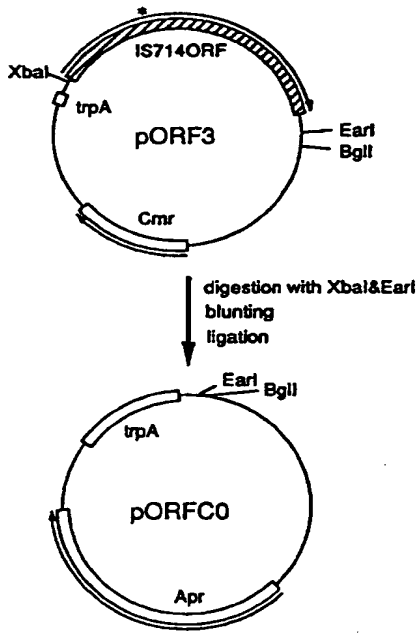

NheI
Cmr
pHIS714
SacII
IS714
SacII
Brevi.TS ori
synthetic DNA
(cloning sites)
EcoRV
digestion with NheI&SacII
ligation
IS714ORF
trpA
pORF3
EarI
BglI
Cmr
BglI
EcoRV
Tn7160
Cmr
KpnI
pHTN7160
Brevi.TS ori
digestion with EarI
blunting
digestion with BglI
digestion with KpnI
blunting
digestion with BglI
recovery of fragment containing
Brevi.TS ori&Tn7160
ligation
EcoRV
Tn7160
Cmr
trpA
pORF41pre
IS714ORF
Brevi.TS ori
Tcr gene fragment(derived from pBR322)
digestion with EcoRI,
blunting
digestion with AvaI,
blunting
digestion with EcoRV
ligation
Cmr
trpA
Tn7162
(Tcr)
IS714ORF
pORF41
Brevi.TS ori

Fig. 14

Fig. 15


IS714ORF
XbaI
trpA
pORF3
EarI
BglI
Cmr
digestion with XbaI&EarI
blunting
ligation
EarI
BglI
trpA
pORFC0
Apr

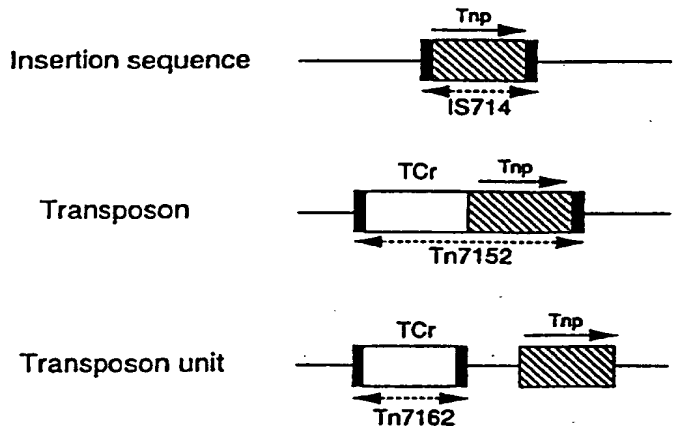
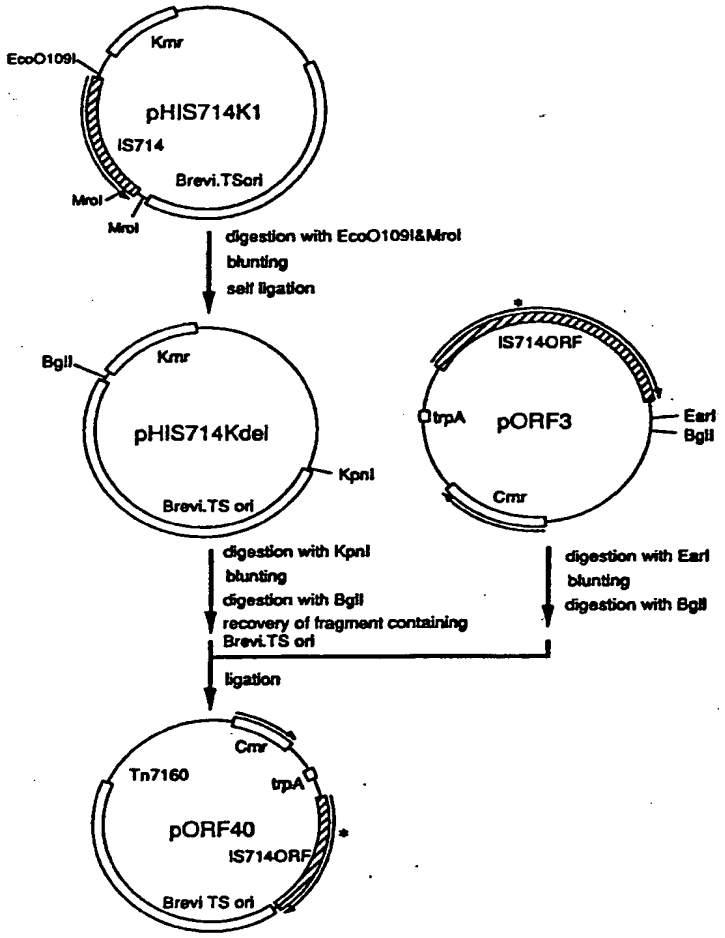

Insertion sequence
Tnp
IS714
Transposon
TCr
Tnp
Tn7152
Transposon unit
TCr
Tnp
Tn7162

Fig. 16


Kmr
EcoO109I
pHIS714K1
IS714
Brevi.TSori
MroI
MroI
digestion with EcoO109I&MroI
blunting
self ligation
BglI
Kmr
pHIS714Kdel
KpnI
Brevi.TS ori
IS714ORF
trpA
pORF3
EarI
BglI
Cmr
digestion with KpnI
blunting
digestion with BglI
recovery of fragment containing
Brevi.TS ori
digestion with EarI
blunting
digestion with BglI
ligation
Cmr
Tn7160
trpA
pORF40
IS714ORF
Brevi TS ori

Fig, 17

Fig. 18

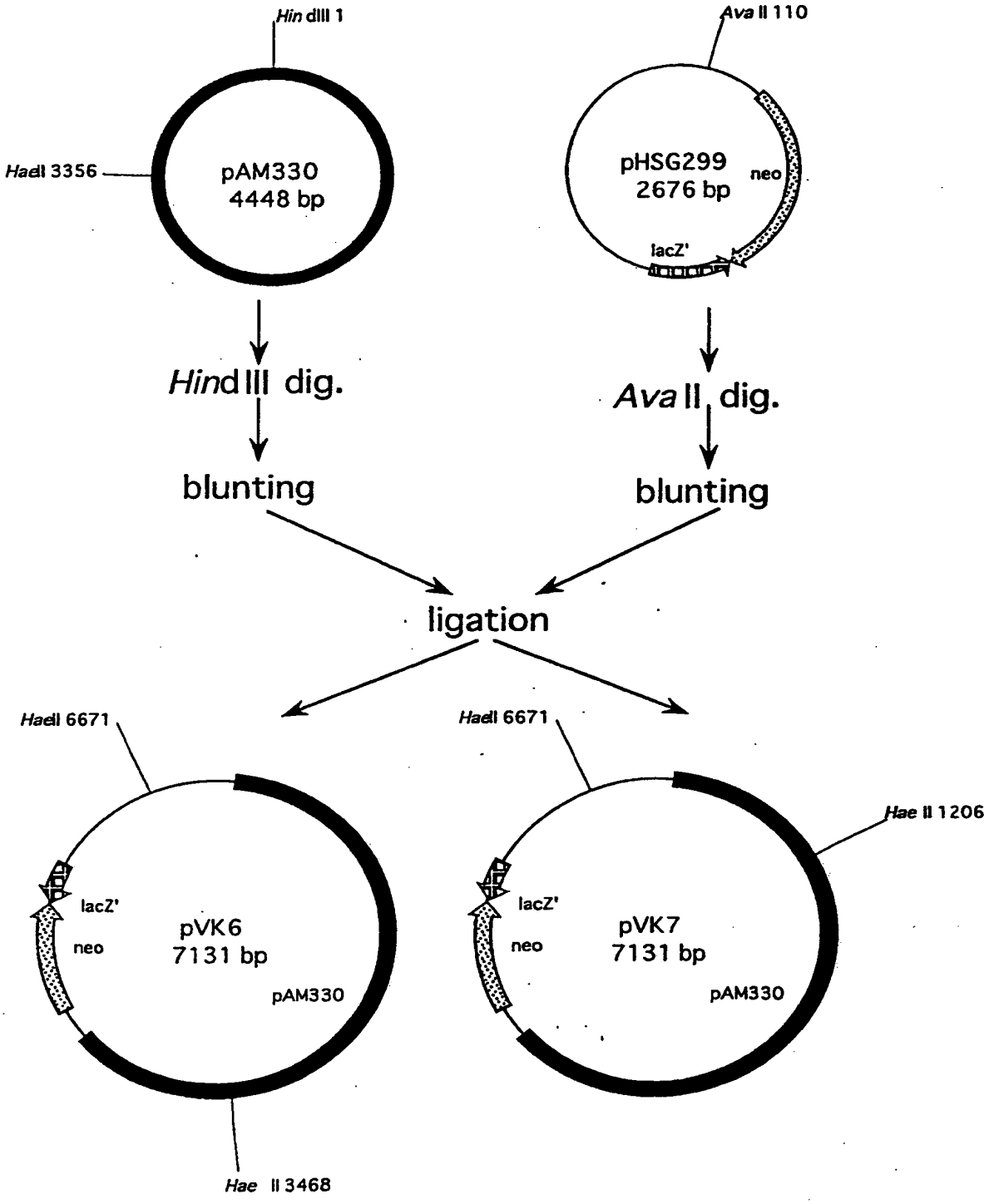

Hin dIII 1
HaeII 3356
pAM330
4448 bp
Ava II 110
pHSG299
2676 bp
neo
lacZ'
HindIII dig.
AvaII dig.
blunting
blunting
ligation
HaeII 6671
HaeII 6671
lacZ'
neo
pVK6
7131 bp
pAM330
lacZ'
neo
pVK7
7131 bp
pAM330
Hae II 1206
Hae II 3468

Fig. 19

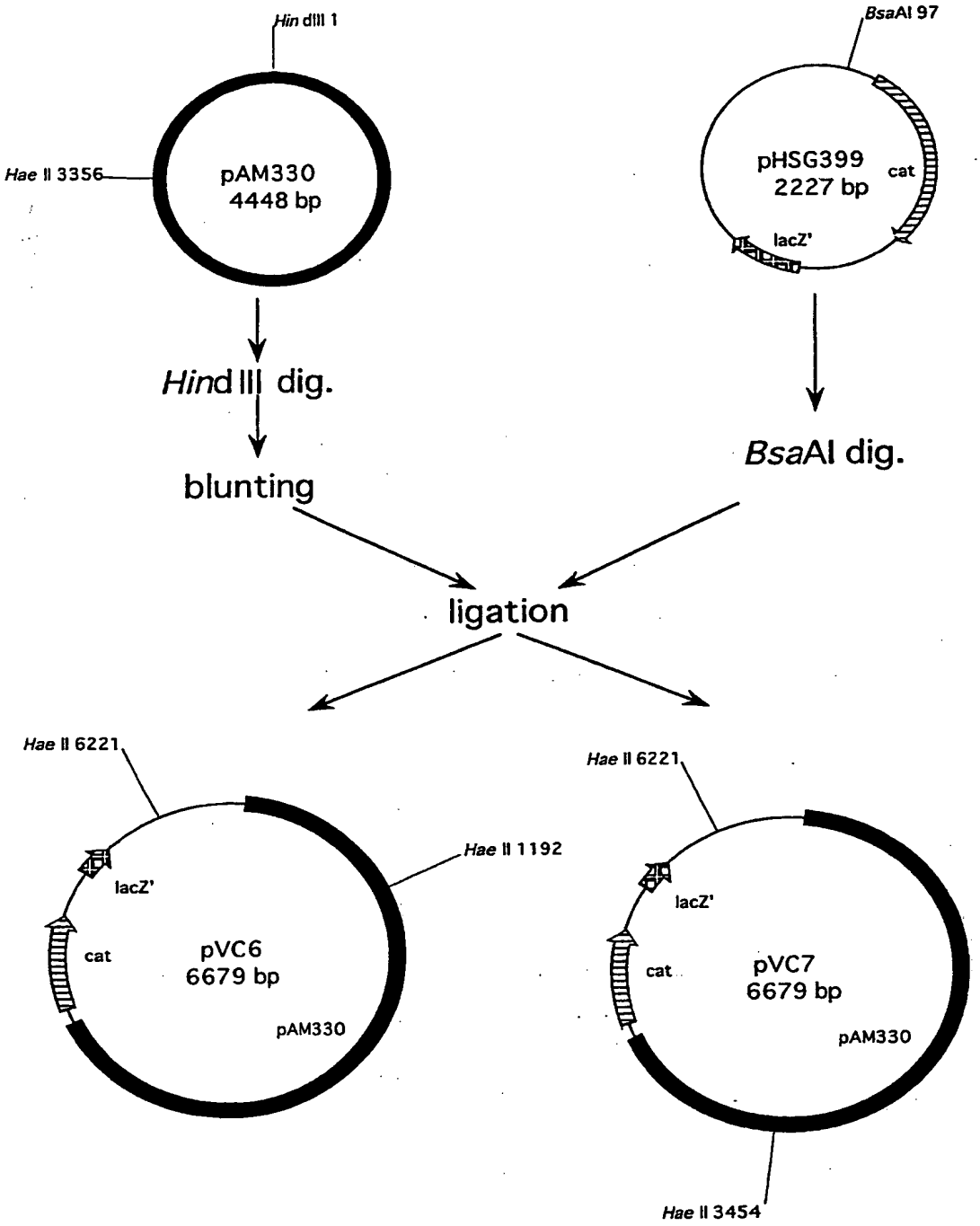

Hin dIII 1
Hae II 3356
pAM330
4448 bp
BsaAI 97
pHSG399
2227 bp
cat
lacZ'
HindIII dig.
blunting
BsaAI dig.
ligation
Hae II 6221
Hae II 1192
lacZ'
cat
pVC6
6679 bp
pAM330
Hae II 6221
lacZ'
cat
pVC7
6679 bp
pAM330
Hae II 3454

FIG. 20

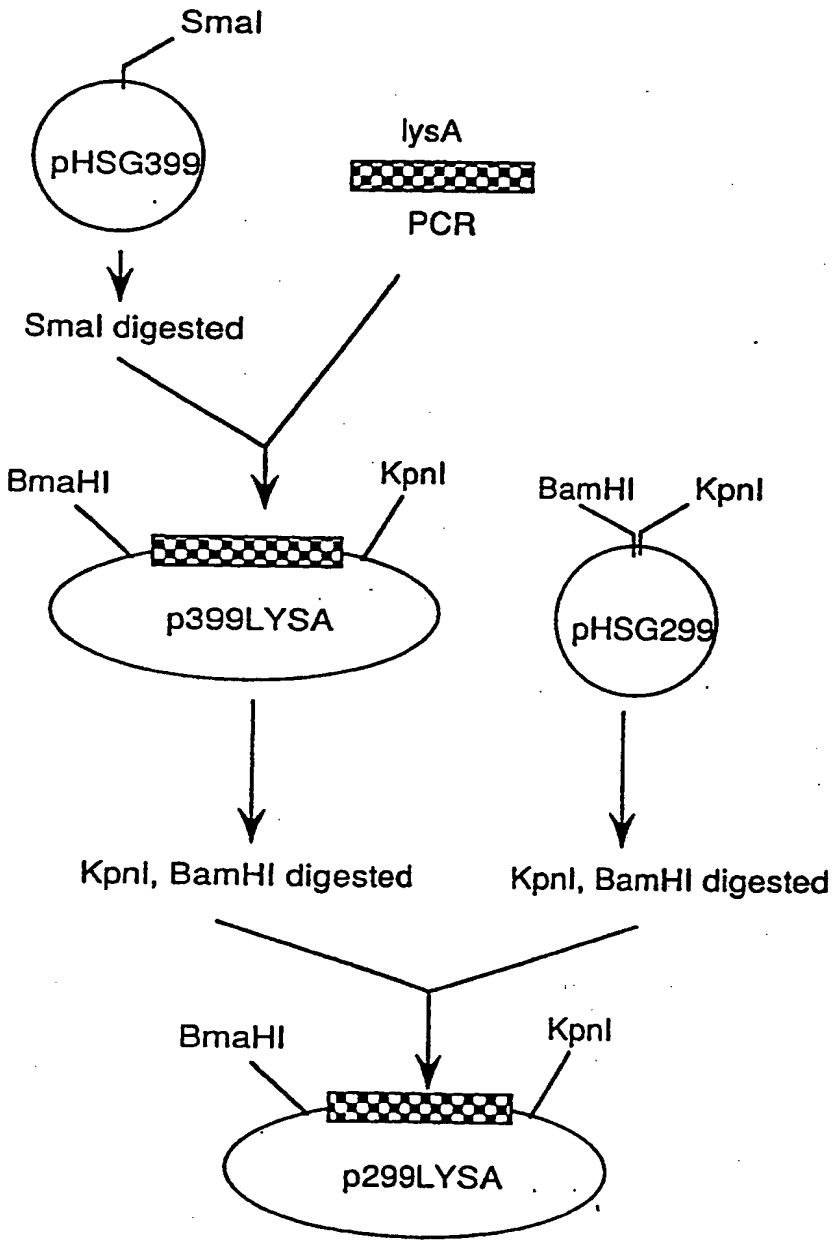

SmaI
pHSG399
lysA
PCR
SmaI digested
BmaHI
KpnI
p399LYSA
BamHI
KpnI
pHSG299
KpnI, BamHI digested
KpnI, BamHI digested
BmaHI
KpnI
p299LYSA

Fig. 21

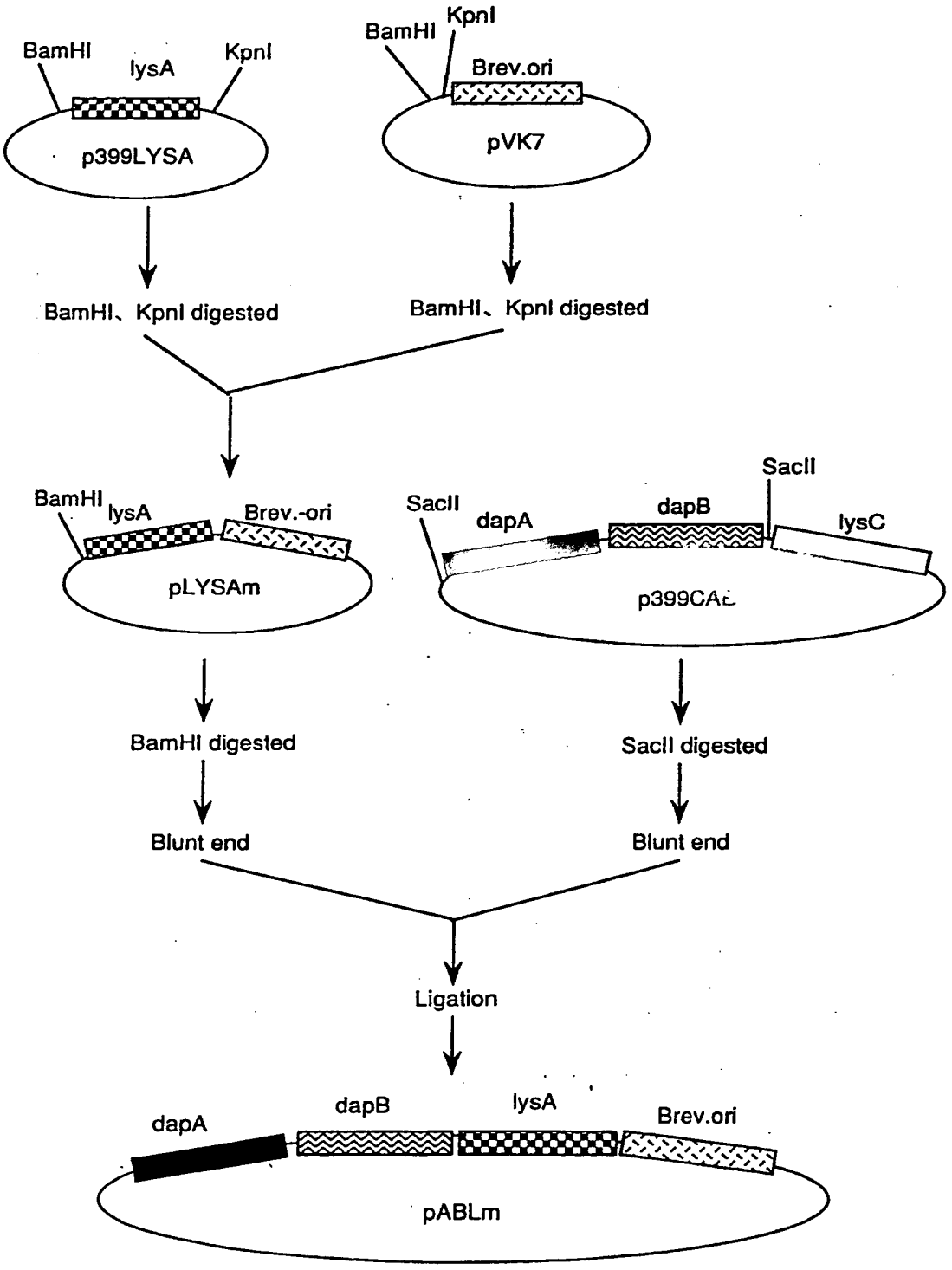

BamHI
lysA
KpnI
p399LYSA
BamHI
KpnI
Brev.ori
pVK7
BamHI、KpnI digested
BamHI、KpnI digested
BamHI
lysA
Brev.-ori
pLYSAm
SacII
dapA
dapB
SacII
lysC
p399CAE
BamHI digested
SacII digested
Blunt end
Blunt end
Ligation
dapA
dapB
lysA
Brev.ori
pABLm

Fig. 22

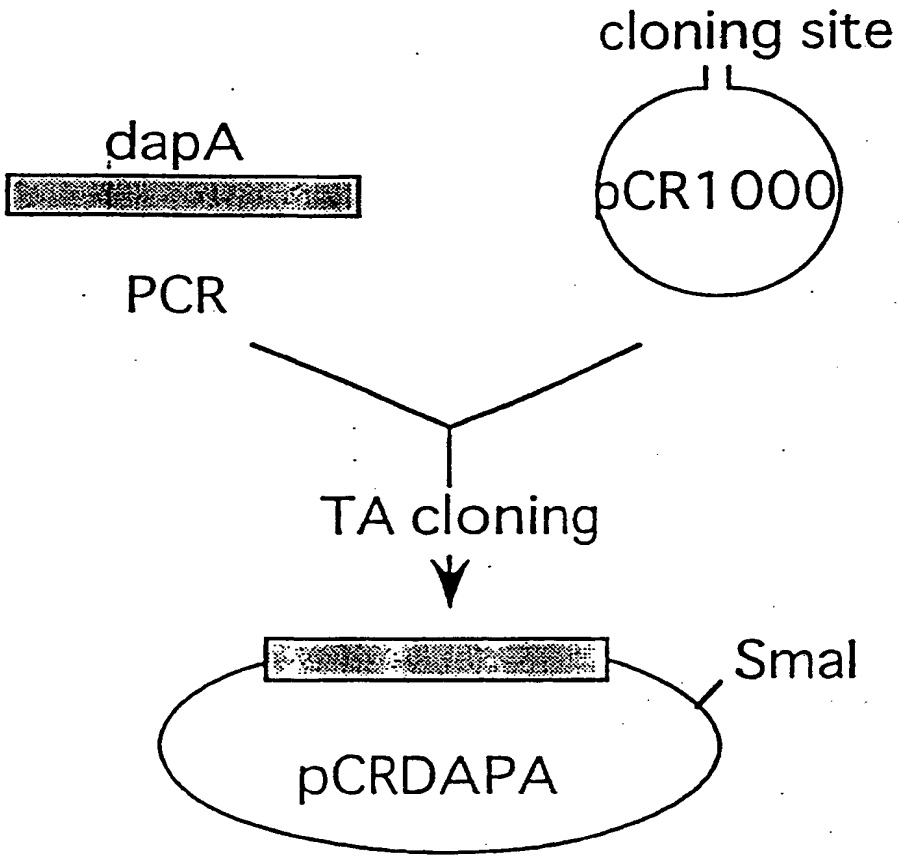


Fig.23

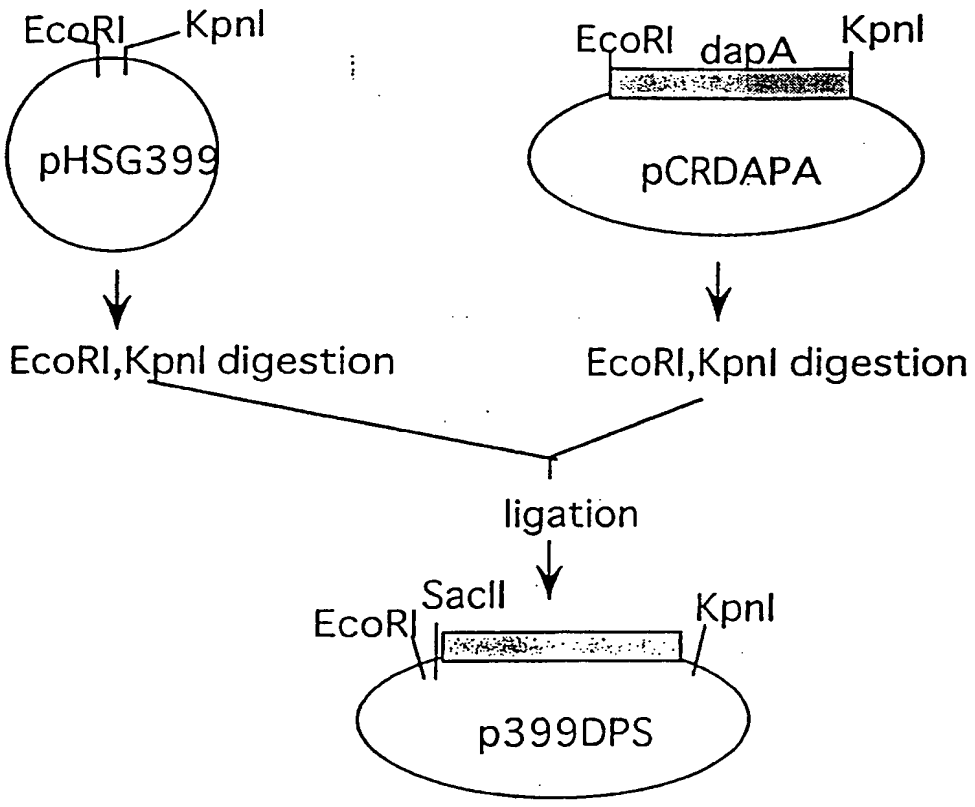

EcoRI
KpnI
pHSG399
EcoRI
dapA
KpnI
pCRDAPA
EcoRI,KpnI digestion
EcoRI,KpnI digestion
ligation
SacII
EcoRI
KpnI
p399DPS

Fig. 24

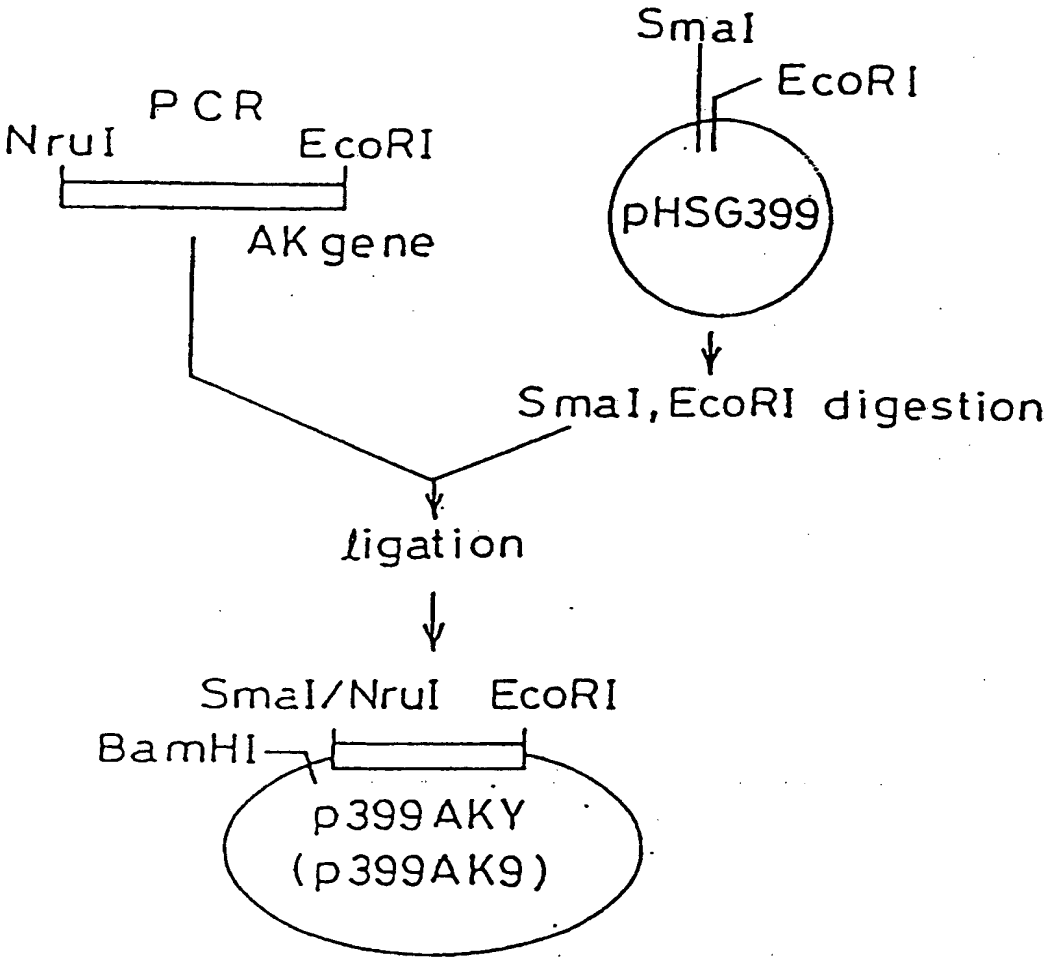

PCR
NruI
EcoRI
AK gene
SmaI
EcoRI
pHSG399
SmaI, EcoRI digestion
ligation
SmaI/NruI
EcoRI
BamHI
p399AKY
(p399AK9)

Fig. 25

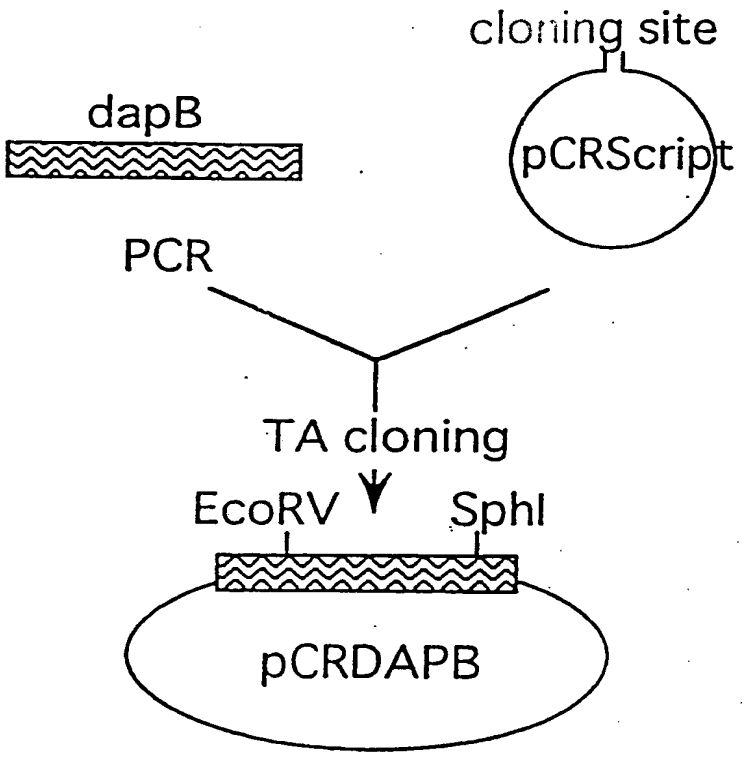

cloning site
dapB
pCRScript
PCR
TA cloning
EcoRV
SphI
pCRDAPB

FIG. 26.

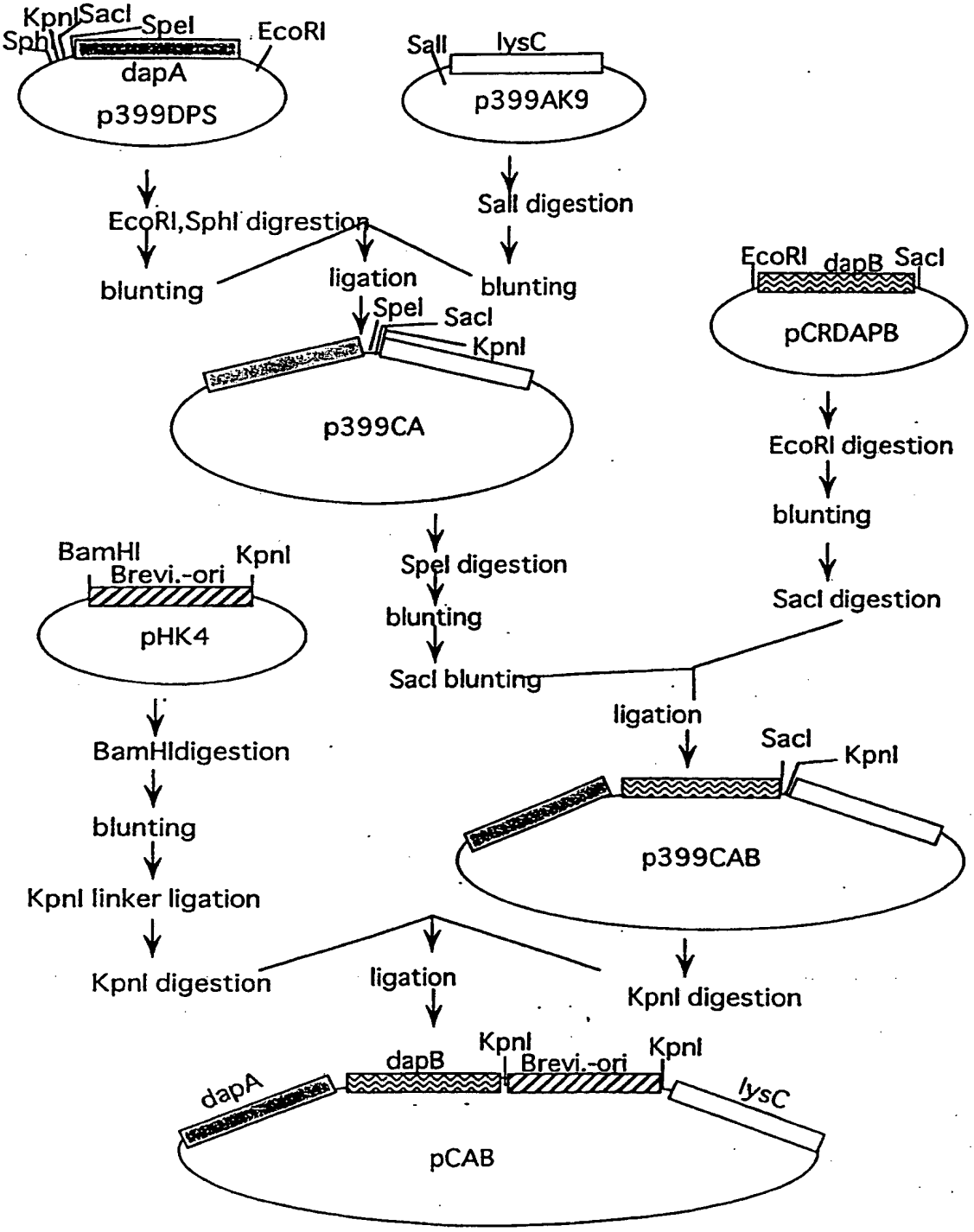

KpnI
SacI
SphI
SpeI
EcoRI
dapA
p399DPS
SalI
lysC
p399AK9
SalI digestion
EcoRI,SphI digrestion
blunting
ligation
blunting
SpeI
SacI
KpnI
p399CA
EcoRI
dapB
SacI
pCRDAPB
EcoRI digestion
blunting
SacI digestion
BamHI
KpnI
Brevi.-ori
pHK4
SpeI digestion
blunting
SacI blunting
ligation
SacI
KpnI
p399CAB
BamHIdigestion
blunting
KpnI linker ligation
KpnI digestion
ligation
KpnI digestion
KpnI
KpnI
dapB
Brevi.-ori
dapA
lysC
pCAB

FIG. 27

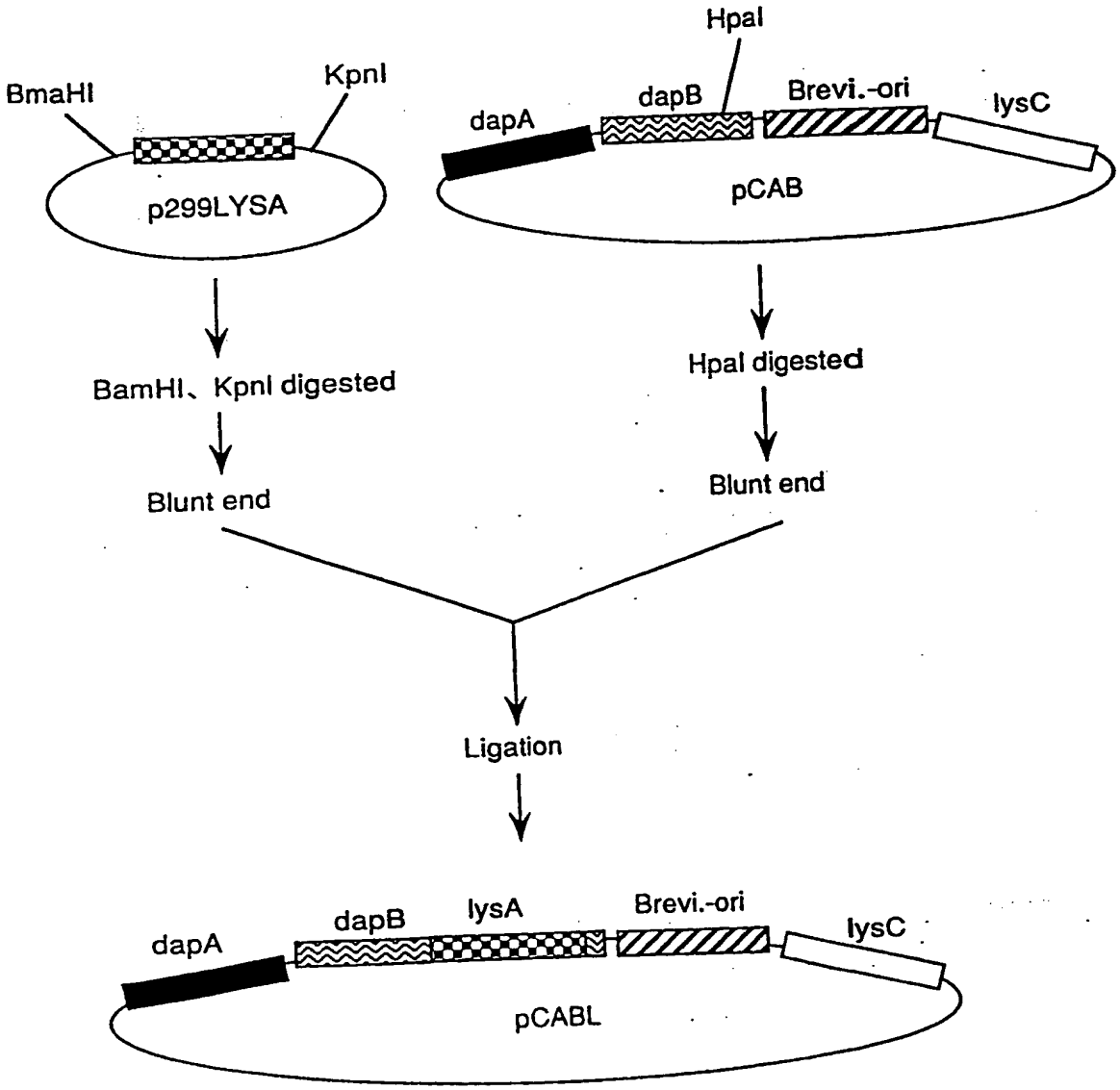

BmaHI
KpnI
p299LYSA
HpaI
dapA
dapB
Brevi.-ori
lysC
pCAB
BamHI、KpnI digested
HpaI digested
Blunt end
Blunt end
Ligation
dapA
dapB
lysA
Brevi.-ori
lysC
pCABL

Fig 28

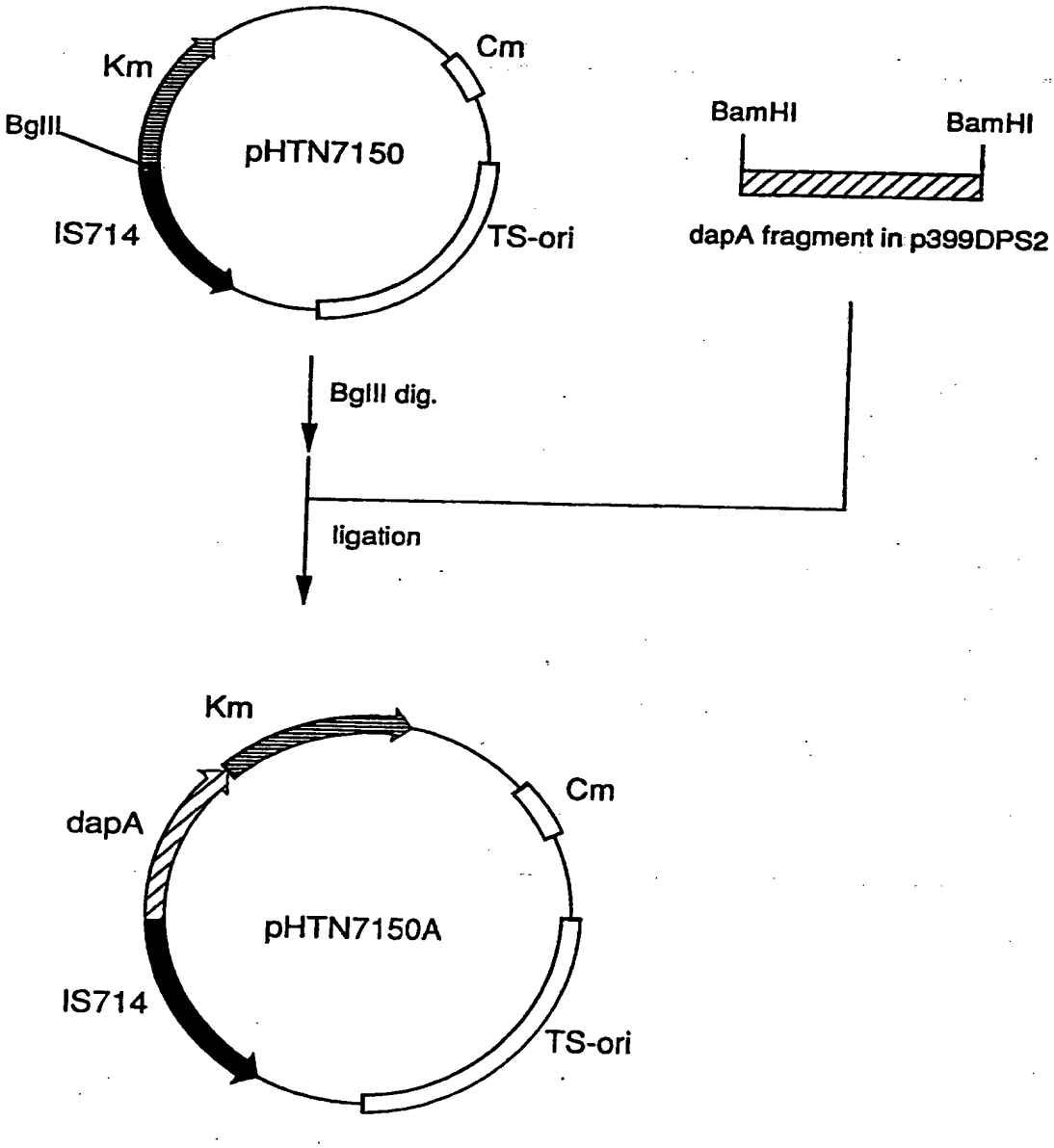
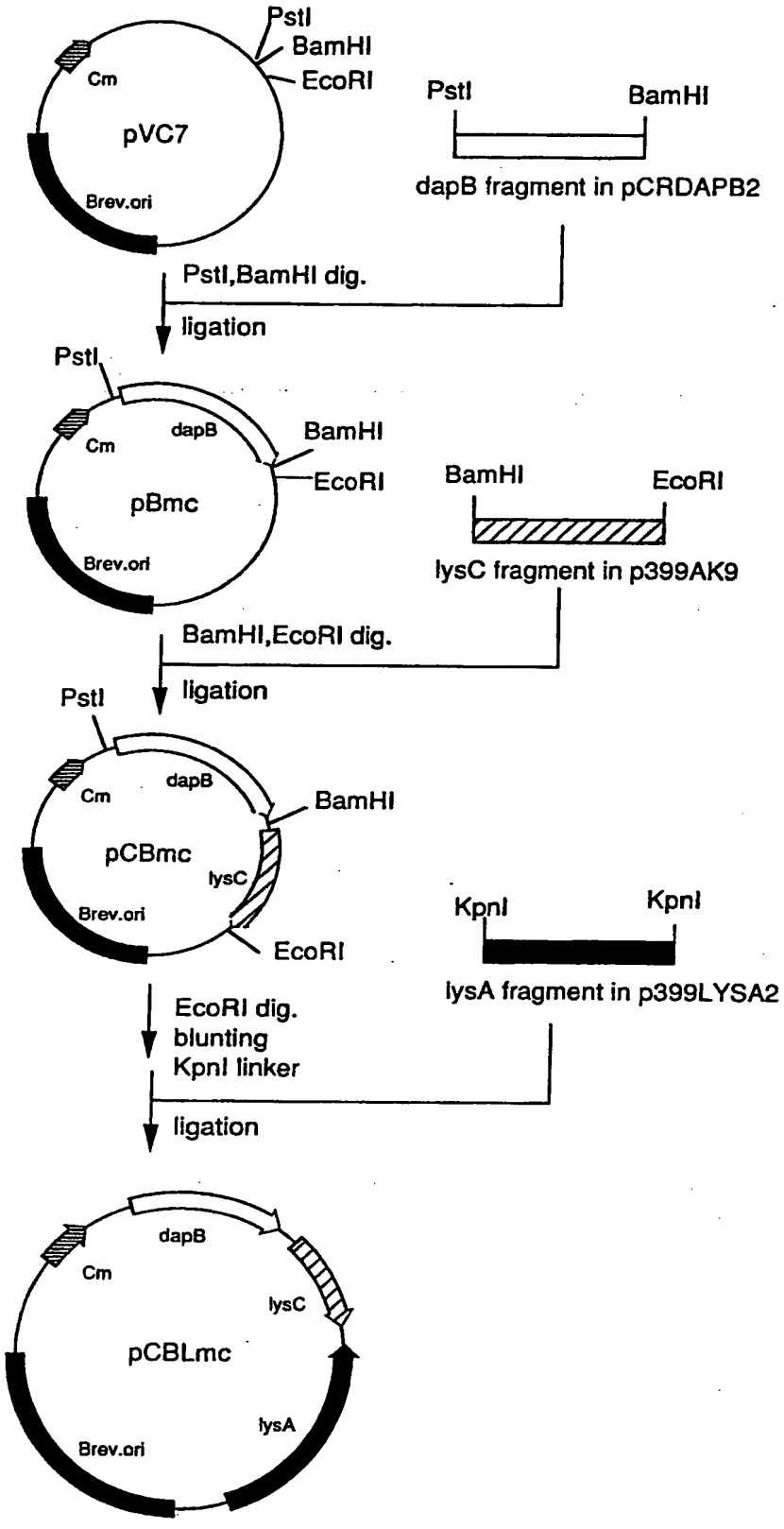

Km
Cm
BglII
pHTN7150
IS714
TS-ori
BamHI
BamHI
dapA fragment in p399DPS2
BglII dig.
ligation
Km
Cm
dapA
pHTN7150A
IS714
TS-ori

Fig.29


PstI
BamHI
EcoRI
Cm
pVC7
Brev.ori
PstI
BamHI
dapB fragment in pCRDAPB2
PstI,BamHI dig.
ligation
PstI
Cm
dapB
BamHI
EcoRI
pBmc
Brev.ori
BamHI
EcoRI
lysC fragment in p399AK9
BamHI,EcoRI dig.
ligation
PstI
Cm
dapB
BamHI
pCBmc
lysC
Brev.ori
EcoRI
KpnI
KpnI
lysA fragment in p399LYSA2
EcoRI dig.
blunting
KpnI linker
ligation
dapB
Cm
lysC
pCBLmc
lysA
Brev.ori

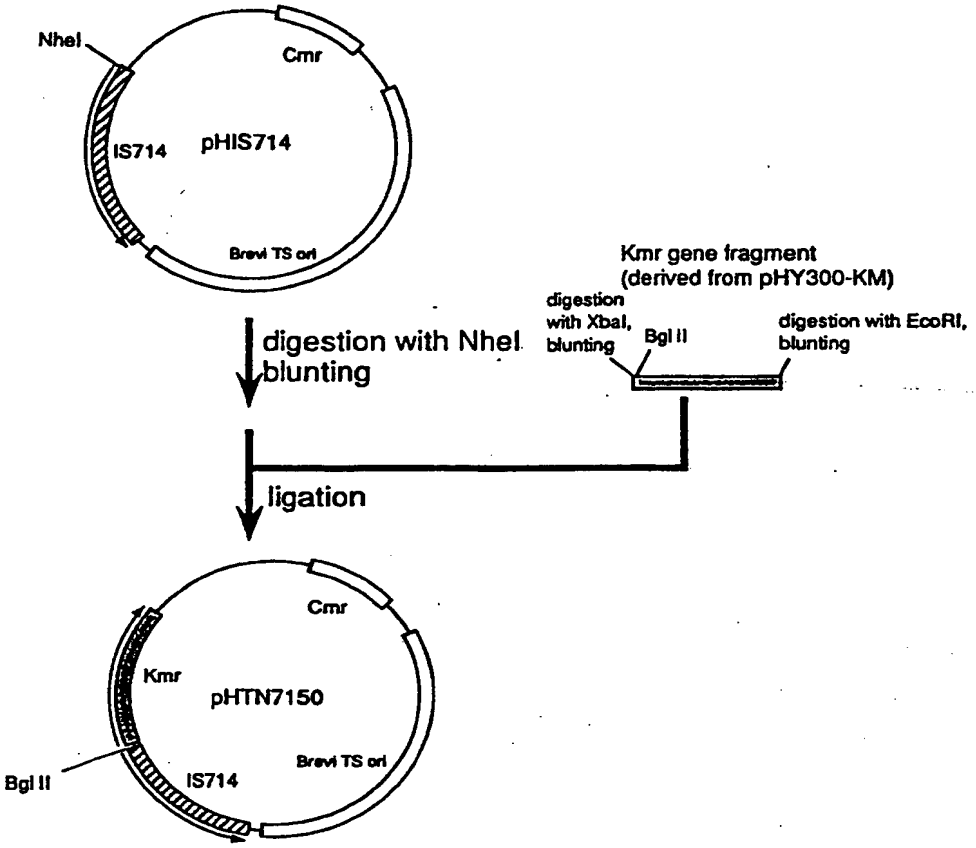

NheI
Cmr
IS714
pHIS714
BrevI TS ori
Kmr gene fragment
(derived from pHY300-KM)
digestion
with XbaI,
blunting
BglII
digestion with EcoRI,
blunting
digestion with NheI
blunting
ligation
Cmr
Kmr
pHTN7150
BrevI TS ori
BglII
IS714

Fig 30.